SELF SELECTION OF DIET

X. APPETITES FOR SODIUM, CHLORIDE AND SODIUM CHLORIDE [1]

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ONE FIGURE

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Although the desire for salt or the salt taste in food has long been recognized in both man and animals, it was Richter ('36) who first showed the relation of salt appetite to the physiologic need for salt. He found that adrenalectomized rats selected more salt solution than normal rats when offered a choice between 3% salt solution and water. Since it was known that high salt intake increases the longevity of adrenalectomized animals, the experiment was indicative of a positive relation between voluntary salt intake and physiologic requirement. These experiments with adrenalectomized animals have been twice confirmed independently (Mark, '42; Clark and Clausen, '43).

It has been recognized that a relation between dietary selection and physiologic need may arise through two mechanisms: (1) the animal may learn that a given choice gives him a subjective feeling of well-being and henceforth he may prefer that choice — this type of appetite has been termed a "learned appetite;" or (2) there may be a direct mechanism relating appetite to need, in which need causes

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an instinctive desire for a given food — this type of appetite would be considered a true hunger. In addition to these, there exist trivial preferences for flavor which may or may not significantly influence experimental results.

Richter preferred the direct mechanism to explain his results. In the first place, he showed that the salt taste was somehow involved, since when he severed the nerves leading to the taste buds the preference for salt was no longer evident. Secondly, when he determined the lowest salt concentration at which rats on a low salt diet showed a preference for salt solution over water, he found that adrenalectomized animals showed a preference for salt at a lower concentration than did normal animals (Richter, '39). He concluded that increased need for salt modified the taste buds in such a way that salt solutions were more readily selected by his animals.

More recently it has appeared that what Richter was measuring was not the true threshold of salt taste but only that salt concentration which the rat found important enough to lead it to modify its drinking habits. Using electrodes on the cut chorda tympani nerve to measure action potentials as an indication of stimulation, Bare ('47) could find no difference in the salt taste thresholds of adrenalectomized and normal rats. Furthermore, Chaplin ('47) has concluded that in rats the salt taste is at least as important in determining salt intake as is salt need, since the daily salt intake of these animals is markedly dependent on the salt concentration of the solutions offered. Richter had found that the results of his experiment with adrenalectomy varied depending on whether a 1% or a 3% salt solution was used.

The present experiments describe the effects of sodium and chloride deficiencies on the preference of rats for sodium chloride and other salts in mixed diets.

EXPERIMENTAL

In the first series of experiments, sodium-deficient and control animals were allowed their choice of a low sodium

diet and one containing sodium chloride. In each experiment 10 albino rats, half males and half females, were weaned at 21 to 25 days of age and fed a low sodium diet [2] in each of two cups. The mineral composition of this diet is shown in table 1. Ten similar animals were fed a diet containing sodium chloride, which varied in amount in the several ex-

TABLE 1

Mineral composition [1] of diets

	LOW SODIUM DIET	LOW CHLORIDE DIET	LOW SODIUM CHLORIDE DIET
	%	%	%
Na	0.002 ± 0.0005 [3]	0.218	0.002 ± 0.0005 [3]
K	0.819	0.449	0.449
Ca	0.610	0.610	0.610
Mg	0.046	0.046	0.046
Fe	0.022	0.022	0.022
Mn	0.005	0.005	0.005
Zn	0.0005	0.0005	0.0005
Cu	0.0005	0.0005	0.0005
Co	0.00002	0.00002	0.00002
Cl	0.339	0.0044 ± 0.0005 [3]	0.0044 ± 0.0005 [3]
P	0.354	0.660	0.354
SO_4	0.229	0.229	0.229
CO_3	0.913	0.913	0.913
I	0.002	0.002	0.002

[1] Composition with respect to minerals other than sodium and chloride was calculated from amount added to the diet.

[2] Determined by Dr. T. S. Danowski, using a flame photometer on solutions of ash.

[3] The procedure for determining chloride was as follows: To a 10-gm sample in a 1000-ml flask (fitted with standard taper joints to a dropping funnel and side arm adapter) were added 100 ml conc. H_2SO_4. The flask was heated slowly until sulfuric acid distilled. The gases produced were lead through a gas washing bottle containing 100 ml 2% silver nitrate in 10% nitric acid. The washing bottle was connected to an aspirator so that the pressure within the system was about 30 mm. After heating, the silver salts were collected by centrifuging, washed, and the silver chloride dissolved in 0.1 N ammonium hydroxide. Silver chloride was reprecipitated in very dilute solution by addition of silver nitrate and nitric acid and determined turbidimetrically.

[2] The low sodium diet contained 10% hydrogenated vegetable oil ("Primex"), 24% vitamin-free casein ("Labco"), 62% sucrose and 4% salt mixture. The salt mixture was that of Jones and Foster ('42) except that the sodium chloride was replaced by potassium chloride.

periments from 0.051 to 12.72%.[3] Vitamins were given separately as pills.[4] During a three-week control period the animals were weighed daily, the food consumed from each cup measured, and the cups then interchanged in a predetermined random manner. During a three-week experimental period both groups of animals were allowed their choice between the low sodium diet and the same sodium chloride-containing diet that the control animals received during the control period. The animals were weighed, food intake from each cup measured and the cups interchanged in the same manner as before.

A very similar experiment was performed in which chloride-deficient animals and control animals were given their choice between chloride-low and sodium chloride-containing diets.[5,6] The mineral content of the chloride-low diet is shown in table 1.

The next series of experiments was designed to test the preferences of sodium-deficient and control animals for diets containing sodium salts other than chloride. The technique was exactly the same except that a low sodium chloride diet, the mineral composition of which is shown in table 1, was used in place of the low sodium diet.[7] A pill containing

[3] The sodium chloride-containing diets were like the sodium-free diet except that potassium chloride was replaced by the proper amounts of sodium chloride. When the amount of sodium chloride desired exceeded that prescribed for the Jones and Foster salt mixture, the additional sodium chloride was added at the expense of the sucrose.

[4] One pill was given each rat daily. It contained: 60 μg thiamine hydrochloride, 120 μg riboflavin, 90 μg pyridoxine hydrochloride, 150 μg calcium pantothenate, 55 I.U. vitamin A and 11 I.U. vitamin D as 0.001 ml Natola, 1 mg α-tocopherol and 10 mg choline chloride, in a dextrin-powdered sugar base.

[5] The diet low in chloride was the same as the low sodium diet except that the Jones and Foster salt mixture was modified by replacing sodium chloride with sodium dihydrogen phosphate.

[6] The vitamin pills were modified in the chloride experiments by the substitution of choline sulfate for choline chloride. The amount of chloride contributed by thiamine and pyridoxine hydrochlorides was negligible.

[7] The low sodium chloride diet was the same as the low sodium diet except that sodium chloride in the Jones and Foster salt mixture was replaced by sucrose. The sodium and chloride contents of this diet were indistinguishable from those found for the low sodium and low chloride diets.

50 mg potassium chloride in powdered sugar was given each rat daily. The sodium-containing diets had 0.05 or 0.1% sodium as various salts added to the low sodium chloride diet, replacing sucrose.

A similar experiment was performed in which the chloride-deficient and control animals were given their choice of a low sodium chloride diet and a diet containing ammonium chloride. In this case a pill containing 50 mg sodium bicarbonate in powdered sugar was given each rat daily. In the ammonium chloride-containing diets, this salt was added to the low sodium chloride diet so that it replaced sucrose.

In previous communications a method was reported which was designed to show, by association with flavor, whether or not appetites for B vitamins were learned (Scott and Verney, '47). The same technique was applied here. Briefly, it consists of allowing an animal, either deficient or normal, an opportunity to associate a flavor or lack of it with a certain food essential in a situation where choice is involved, and then of changing the choice so that the flavor is in the opposite relation to the essential. Association of flavor with the food essential is shown by eating behavior when the change is made, and is evidence that the appetite is learned. For a full discussion, our earlier papers should be consulted.

In the case of sodium chloride and sodium deficiency, the detailed experiment was as follows: 20 animals were divided into 4 groups, each of which received one of the following diets in both cups during the control period; (1) low sodium diet; (2) flavored low sodium diet;[8] (3) sodium chloride-containing (0.05%) diet; and (4) flavored sodium chloride-containing diet. Body weight and food intake were measured as before, and the cups interchanged.

The experimental period was divided into 4 parts of 5, 5, 5 and 6 days. During the first and third parts, half the deficient and half the control animals received a choice of the above diets (1) and (4) while the others received a

[8] All flavored diets contained 10 p.p.m. oil of anise, which is neither preferred nor avoided by rats (Scott and Quint, '46a, b).

TABLE 2

Appetite for sodium chloride[1]

NaCl in choice	Animals	3-week control period			3-week experimental period				
		Body weight change	Food eaten	Average NaCl intake	Body weight change	Food eaten	Average NaCl intake	Appetite for NaCl[2]	Appetite attributable to deficiency
%		*gm*	*gm*	*mg/day*	*gm*	*gm*	*mg/day*	%	%
					Sodium-deficient				
0.051	Deficient	27.5 ± 7.1	118.6 ± 9.4	0.3	67.0 ± 6.0	223.9 ± 15.9	5.3	44.1 ± 5.5	
	Control	58.5 ± 3.8	149.5 ± 7.2	4.0	66.7 ± 5.1	262.9 ± 11.0	5.8	36.3 ± 7.3	7.8 ± 9.1
0.127	Deficient	33.3 ± 3.9	148.4 ± 5.4	0.4	85.7 ± 7.0	243.4 ± 12.2	10.7	15.0 ± 4.8	
	Control	79.5 ± 3.5	197.4 ± 7.9	12.4	71.0 ± 8.5	278.4 ± 12.8	11.4	13.0 ± 4.2	2.0 ± 6.4
0.255	Deficient	30.9 ± 3.4	128.2 ± 6.6	0.3	81.9 ± 3.7	223.5 ± 8.8	14.5	— 0.3 ± 12.4	
	Control	72.6 ± 2.9	171.6 ± 4.9	21.2	66.4 ± 4.1	260.8 ± 7.4	20.2	13.2 ± 10.0	— 13.5 ± 15.9
0.508	Deficient	40.9 ± 3.4	122.7 ± 6.3	0.3	83.6 ± 5.5	236.2 ± 14.6	28.4	— 2.4 ± 8.6	
	Control	67.9 ± 3.5	144.9 ± 6.2	35.4	70.2 ± 6.4	242.5 ± 13.6	34.5	9.2 ± 7.6	— 11.6 ± 11.5
1.27	Deficient	43.3 ± 3.9	134.5 ± 4.6	0.3	85.0 ± 6.1	249.7 ± 5.2	95.1	14.8 ± 5.9	
	Control	81.7 ± 4.1	161.9 ± 8.7	97.9	65.2 ± 6.1	253.7 ± 14.8	103.4	19.5 ± 11.8	— 4.7 ± 13.2
2.55	Deficient	30.8 ± 3.4	124.7 ± 5.1	0.3	60.0 ± 3.4	185.9 ± 7.4	86.7	— 22.2 ± 8.5	
	Control	56.0 ± 2.7	143.6 ± 7.5	174.4	44.1 ± 3.3	188.8 ± 8.8	98.1	— 2.3 ± 10.7	— 19.9 ± 13.7
5.08	Deficient	40.4 ± 4.2	141.4 ± 5.1	0.3	70.8 ± 5.1	221.7 ± 7.5	193.1	— 10.6 ± 6.8	
	Control	70.0 ± 5.3	172.5 ± 8.8	417.7	62.1 ± 7.7	240.6 ± 14.3	185.7	— 21.4 ± 4.5	10.8 ± 8.2
12.72	Deficient	45.1 ± 3.0	187.6 ± 6.7	0.4	85.5 ± 5.4	273.6 ± 12.0	347.1	— 28.2 ± 3.9	
	Control	58.4 ± 2.8	215.7 ± 12.2	1307.3	68.4 ± 5.6	292.3 ± 12.1	411.5	— 26.7 ± 3.3	— 1.5 ± 5.1
					Chloride-deficient				
0.28	Deficient	46.4 ± 2.1	142.2 ± 5.9	0.5	66.3 ± 4.6	224.8 ± 7.6	16.4	9.1 ± 6.2	
	Control	81.3 ± 3.4	171.7 ± 4.5	23.7	58.2 ± 4.3	249.7 ± 7.2	15.2	— 2.6 ± 4.8	11.7 ± 7.8

[1] All data are given in terms of mean and standard error of the mean.

[2] The figures represent per cent of the sodium chloride-containing diet eaten during the experimental period minus the per cent of food eaten from the corresponding cup during the control period. A positive number is an appetite, a negative one an avoidance.

choice of diets (2) and (3). During the second and 4th parts, those animals who chose between (1) and (4) previously now had choices between (2) and (3), while those previously choosing between (2) and (3) were given (1) and (4). Again body weights and food consumption were determined daily and the cups interchanged.

The effect of flavor on appetites for other sodium salts was determined in the same manner, with suitable alteration in diets and with supplementation by potassium chloride pills. All sodium-containing diets had 0.05% sodium present. The effect of flavor on appetite for ammonium chloride required, again, suitable diets and supplementation with sodium bicarbonate pills. The ammonium chloride diet contained 0.2% chloride.

RESULTS

As may be seen in table 2, it was found that there was no consistent or significant difference between sodium-deficient and control animals when their choices between low sodium and sodium chloride-containing diets were compared. At very low sodium chloride concentrations both groups preferred the sodium chloride-containing to the low sodium diets, and at very high concentrations the diets were probably too salty to be acceptable. Over a range of intermediate concentrations, from 0.25 to 2.5% sodium chloride, there was no difference between the acceptability of the low sodium and the sodium chloride-containing diets. The one experiment on chloride-deficient animals gave a result consistent with these conclusions.

The attempts to show appetites for other sodium salts are summarized in table 3. In these experiments, only in the case of sodium bicarbonate was a greater appetite for sodium shown by deficient than by control animals, although the result with sodium dihydrogen phosphate is equivocal. The preference of chloride-deficient animals for ammonium chloride-containing diets was greater than that of the controls, but its significance is doubtful.

TABLE 3

Appetites for sodium and chloride

SALT IN CHOICE	ANIMALS	3-WEEK CONTROL PERIOD			3-WEEK EXPERIMENTAL PERIOD				
		Body weight change	Food eaten	Average Na (or Cl) intake	Body weight change	Food eaten	Average Na (or Cl) intake	Appetite for Na (or Cl)	Appetite attributable to deficiency
%		*gm*	*gm*	*mg/day*	*gm*	*gm*	*mg/day*	%	%
0.05% Na as NaH_2PO_4	Na Deficient	30.6 ± 2.5	127.4 ± 3.2	0.1	57.9 ± 4.6	229.4 ± 8.1	3.2	9.0 ± 9.9	
	Control	62.1 ± 3.5	162.1 ± 6.2	4.0	55.5 ± 7.1	248.6 ± 12.7	3.0	−9.8 ± 9.3	18.8 ± 13.6
0.1% Na as NaH_2PO_4	Na Deficient	44.6 ± 4.3	137.8 ± 7.9	0.1	74.4 ± 4.8	235.1 ± 6.7	5.6	−0.6 ± 6.8	
	Control	66.2 ± 2.2	148.8 ± 7.1	7.2	52.9 ± 7.7	224.7 ± 8.1	5.1	0.3 ± 7.3	−0.9 ± 10.0
0.05% Na as $NaHCO_3$	Na Deficient	25.9 ± 3.1	118.6 ± 5.8	0.1	72.7 ± 4.6	202.8 ± 10.4	3.3	9.7 ± 7.5	
	Control	62.3 ± 3.6	150.2 ± 4.8	3.7	62.6 ± 5.5	235.3 ± 9.6	2.2	−23.5 ± 6.1	33.2 ± 9.0
0.05% Na as Na_2SO_4	Na Deficient	34.6 ± 4.0	107.2 ± 7.4	0.1	63.2 ± 5.0	198.1 ± 9.4	3.1	13.7 ± 5.6	
	Control	62.0 ± 3.3	135.1 ± 4.5	3.3	45.6 ± 3.6	194.8 ± 7.3	2.8	6.6 ± 5.1	7.1 ± 7.5
0.2% Cl as NH_4Cl	Cl Deficient	39.5 ± 2.3	121.5 ± 3.5	0.3	81.5 ± 5.2	232.0 ± 10.5	17.0	32.5 ± 8.4	
	Control	57.5 ± 2.2	121.4 ± 3.6	14.1	69.5 ± 4.8	224.7 ± 8.5	12.1	4.8 ± 11.8	27.7 ± 14.5

TABLE 4

Effect of flavor on appetites for sodium and chloride

ANIMALS	3-WEEK CONTROL PERIOD		3-WEEK EXPERIMENTAL PERIOD			
	Body weight change	Food eaten	Body weight change	Food eaten	Appetite for sodium (or chloride)	Per cent eaten associated with flavor [1]
	gm	*gm*	*gm*	*gm*	%	
			Sodium chloride			
Na deficient	39.7 ± 4.5	120.8 ± 9.1	67.8 ± 5.4	206.3 ± 14.5	14.4 ± 9.8	54.5 ± 5.7
Control	63.7 ± 4.4	148.0 ± 9.9	62.8 ± 6.8	223.1 ± 14.7	5.9 ± 5.1	50.9 ± 8.2
			Sodium bicarbonate			
Na deficient	39.6 ± 2.8	151.5 ± 5.8	73.6 ± 6.0	247.8 ± 8.3	2.6 ± 4.9	57.5 ± 5.1
Control	65.5 ± 5.3	169.8 ± 9.7	66.1 ± 6.7	255.2 ± 12.3	1.0 ± 6.9	55.8 ± 3.9
			Sodium sulfate			
Na deficient	47.1 ± 3.4	141.1 ± 4.2	78.7 ± 7.3	240.5 ± 7.9	6.1 ± 4.1	31.1 ± 6.1
Control	63.0 ± 7.3	155.4 ± 9.0	57.4 ± 6.0	236.6 ± 16.9	1.4 ± 3.9	46.9 ± 6.7
			Sodium dihydrogen phosphate			
Na deficient	35.8 ± 1.8	128.9 ± 6.5	63.1 ± 5.7	179.8 ± 8.4	−2.4 ± 7.1	41.4 ± 3.7
Control	69.7 ± 5.2	153.0 ± 9.6	58.4 ± 2.6	212.6 ± 9.9	−0.3 ± 4.5	49.7 ± 4.1
			Ammonium chloride			
Cl deficient	30.9 ± 2.3	129.4 ± 4.8	57.4 ± 3.6	210.3 ± 7.8	−7.8 ± 3.2	50.4 ± 5.3
Control	45.2 ± 2.4	136.8 ± 4.6	50.6 ± 3.1	210.9 ± 7.1	8.2 ± 6.9	51.7 ± 4.4

[1] Per cent eaten of that food, flavored or unflavored, which first contained sodium or chloride in the experimental period. A result significantly greater than 50 is evidence of association of the food essential with flavor.

The results of the flavor experiments are shown in table 4 and figure 1. In no case was the alternation of choice when flavor was changed similar to that observed with B vitamins.

DISCUSSION

Sodium chloride is unusual in that it is the one dietary essential that is universally recognizable through its unique flavor. It has been and is exceedingly difficult to determine whether preference for salt flavor is: (1) caused by need

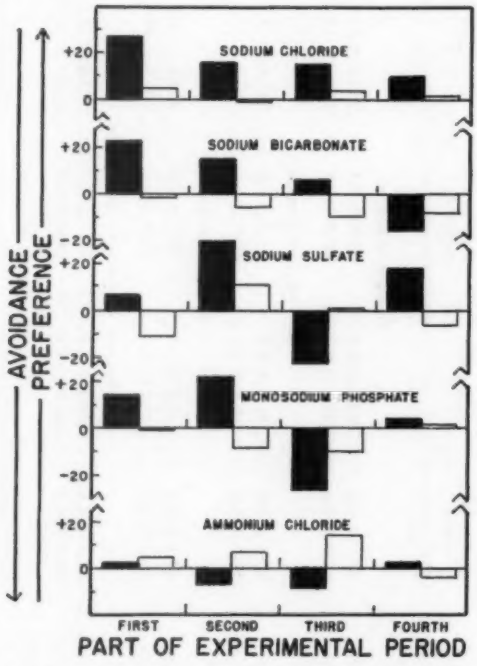

Fig. 1 Effect of flavor on appetites for foods containing sodium or chloride. Flavor was alternated with respect to the mineral between each part of the experimental period. Preference (positive) or avoidance (negative) was calculated as per cent eaten of sodium or chloride containing-food minus 50. Solid bars = average of 10 deficient animals; open bars = average of 10 normal animals.

for salt; (2) learned as a result of the beneficial effects of salt on well-being; or (3) a flavor effect, wholly unrelated to salt need. However, if a change in salt need produces a change in salt appetite, then either the first or second explanation or both, must be operative, but this does not mean that the third is without influence.

In general, these results indicate that sodium-deficient rats do not show a greater preference for sodium-containing diets than do control animals. If the experiments on sodium deficiency shown in tables 2 and 3 are considered together, the preference of 120 deficient animals for sodium-containing diets was only $2.3 \pm 3.1\%$ greater than that of 120 control animals. In disagreement with this generalization is the appetite shown for sodium bicarbonate, which has a high probability of being significant. No reasonable explanation for this disagreement is evident.

On the basis of the experiments on adrenalectomy there can be little doubt that change in salt need in that case leads to a change in salt appetite. Sodium deficiency, however, appears not to lead to a change in salt appetite, and this may be due to lack of motivation in the latter case. It is possible that sodium-deficient animals recover from deficiency before a habit of eating the sodium-containing food is established. Evidence for this hypothesis was obtained from a study of food choices during the first week of the experimental period, with results shown in table 5. While it is true that significantly greater appetites were shown by deficient animals only in the cases of sodium bicarbonate and one concentration of sodium chloride, 120 deficient animals chose $12.6 \pm 3.3\%$ more sodium-containing diet than did 120 control animals during the first week of the experimental period, when the effects of deficiency would be expected to be most evident.

The experiments with flavor are inconclusive. In our experiments with B vitamins we in no case found any evidence that the flavor of the vitamin itself was ever recognized by the animal. In the present experiments it is certainly possible

that the flavor of the sodium salts affected the results. If the flavor of the sodium salt were recognizable in the diet, there is no reason to believe that an animal would be more likely to associate any beneficial effect of the diet with anise flavor than with the flavor of the sodium salt.

TABLE 5

Initial appetites for sodium salts

SODIUM IN CHOICE	APPETITE FOR SODIUM [1] OF Deficient anima's	Control anima's	APPETITE ATTRIBUTABLE TO DEFICIENCY
%	%	%	%
	Sodium chloride		
0.02	56.9 ± 5.5	39.5 ± 8.2	17.4 ± 9.9
0.05	34.7 ± 4.5	18.0 ± 6.0	16.7 ± 7.5
0.10	13.2 ± 10.8	9.4 ± 3.3	3.8 ± 11.3
0.20	15.2 ± 7.9	9.1 ± 7.5	6.1 ± 10.8
0.50	13.9 ± 6.8	11.9 ± 15.0	2.0 ± 16.5
1.00	−34.2 ± 10.8	−21.1 ± 8.8	−13.1 ± 13.9
2.00	−9.6 ± 4.5	−24.0 ± 8.9	14.4 ± 10.0
5.00	−26.6 ± 6.2	−23.5 ± 3.1	−3.1 ± 7.0
	Sodium dihydrogen phosphate		
0.05	11.9 ± 9.7	−14.4 ± 12.0	26.3 ± 15.4
0.10	21.0 ± 8.7	8.4 ± 7.7	12.6 ± 11.6
	Sodium bicarbonate		
0.05	22.6 ± 9.0	−27.7 ± 6.5	50.3 ± 11.1
	Sodium sulfate		
0.05	15.5 ± 6.6	−0.1 ± 5.9	15.6 ± 8.9

[1] Figures represent per cent of sodium-containing diet eaten during the first week of the experimental period minus the per cent of food eaten from the corresponding cup during the last week of the control period.

Two of the salts, bicarbonate and chloride, gave results which could be interpreted as indicating that the animals were influenced in their choice by the flavor of the salt and not of the anise, while the other two did not. In the cases of phosphate and sulfate it will be noted that, excluding the first part of the experimental period, the usual change of appetite associated with change in flavor is observed. A

possible explanation of these results may be that it took longer than the 5-day first part of the experimental period for the beneficial effects of sodium to become associated with flavor or lack of it, but this explanation is inconsistent with the transient nature of sodium appetites indicated above.

In conclusion, it appears that the possibility of demonstrating an appetite for sodium-containing diets in sodium-deficient rats is not an encouraging one. Under very special conditions an appetite may be found, but it is vague and indefinite when compared with similar appetites in rats for B vitamins. Furthermore, the results obtained seem to depend to a large extent on which particular salt of sodium is used, and therefore conceivably on the flavor of the salt.

SUMMARY

Both normal and sodium-deficient rats showed no preference for diets containing from 0.1% to 2.5% sodium chloride over low sodium diets. At lower levels, both groups preferred sodium chloride-containing diets and at higher levels both groups avoided them. An appetite for sodium bicarbonate, but not for sodium dihydrogen phosphate or sodium sulfate, was found in sodium-deficient rats. Results of tests for an appetite for chloride were equivocal.

LITERATURE CITED

BARE, J. K. 1947 An experimental investigation of the specific hunger for sodium chloride exhibited by the rat. Doctoral thesis, Brown University. Cited by Young, below.

CHAPLIN, J. P. 1947 Preferences of adrenalectomized rats for salt solutions of different concentrations. Doctoral thesis, University of Illinois. Cited by Young, below.

CLARK, W. G., AND D. F. CLAUSEN 1943 Dietary "self-selection" and appetite of untreated and treated adrenalectomized rats. Am. J. Physiol., *139:* 70.

JONES, J. H., AND C. FOSTER 1942 A salt mixture for use with basal diets either low or high in phorphorus. J. Nutrition, *24:* 245.

MARK, J. 1942 Inactivation of desoxycorticosterone acetate. Endocrinology, *31:* 582.

RICHTER, C. P. 1936 Increased salt appetite in adrenalectomized rats. Am. J. Physiol., *115:* 155.

———— 1939 Salt taste thresholds of normal and adrenalectomized rats. Endocrinology, *24:* 367.

SCOTT, E. M., AND E. QUINT 1946a Self selection of diet. II. The effect of flavor. J. Nutrition, *32:* 113.

———— 1946b Self selection of diet. III. Appetites for B vitamins. Ibid., *32:* 285.

SCOTT, E. M., AND E. L. VERNEY 1947 Self selection of diet. VI. The nature of appetites for B vitamins. Ibid., *34:* 471.

YOUNG, P. T. 1948 Appetite, palatability and feeding habit: A critical review. Psychol. Bull., *45:* 289.

SELF SELECTION OF DIET

XI. APPETITES FOR CALCIUM, MAGNESIUM AND POTASSIUM [1]

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TWO FIGURES

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Although it is a popular belief that children and pregnant women may eat chalk or plaster because they need calcium, Richter and Eckert ('37) were the first to show that an appetite for calcium may be related to need for that element. They found that rats after parathyroidectomy had an increased appetite for 2.4% calcium lactate. No experiments on appetites for magnesium and potassium have heretofore been reported.

The present paper compares the appetites of mineral-deficient and control animals for diets containing calcium, magnesium, and potassium.

EXPERIMENTAL

The first series of experiments were concerned with the choice of compounds of calcium in mixed diets by calcium-deficient and normal animals. In each experiment weanling albino rats were divided into two groups, one of which re-

[1] This research was aided by grants from the Nutrition Foundation, Inc., and the Buhl Foundation.

ceived a low calcium diet [2] and the other a calcium-containing diet [3] in each of two cups for a period of three weeks. The mineral composition of the low calcium diet is shown in table 1. Vitamins were fed separately as pills.[4] Throughout this control period the amount of food eaten from each cup was determined daily, the animals weighed, and the cups then interchanged in a predetermined random manner.

During the three-week experimental period which followed, both groups were given their choice in separate cups between the low calcium diet and the same calcium-containing diet given the control animals during the control period. The amount of each diet eaten was determined daily, the animals weighed, and the cups interchanged as before.

In the second series of experiments the same type of study was conducted with magnesium. The only differences were in the diets,[5,6] and in the length of the experiment. It was found necessary to limit the control period to two weeks, since mortality among magnesium-deficient animals was high during the third week. The experimental period was also of two weeks' duration.

Two experiments were performed to determine the behavior of potassium-deficient and control animals in selecting potassium-containing diets. The technique was the same as

[2] The low calcium diet contained 24% casein ("Labco, vitamin-free"), 10% hydrogenated vegetable oil ("Primex"), 62% sucrose, and 4% salt mixture. The salt mixture was that of Jones and Foster ('42), modified by replacement of calcium carbonate with sucrose.

[3] Calcium-containing diets were made by replacing sucrose in the low calcium diet by the appropriate amount of calcium salt.

[4] One pill was given each rat daily. It contained approximately 60 μg thiamine hydrochloride, 120 μg riboflavin, 90 μg pyridoxine hydrochloride, 150 μg sodium pantothenate, 55 I.U. vitamin A and 11 I.U. vitamin D as 0.001 ml Natola, 1 mg α-tocopherol, and 10 mg choline chloride, in a dextrin-powdered sugar base.

[5] The low magnesium diet was the same as the low calcium diet except for the salt mixture. The usual mixture of Jones and Foster was modified by replacement of magnesium sulfate with sucrose.

[6] Magnesium-containing diets were formulated by replacing sucrose in the low magnesium diet by appropriate amounts of magnesium compounds.

above, although the diets [7] differed. The second of these experiments was limited to two-week control and experimental periods because of the early mortality of the potassium-deficient animals.

The salts of calcium and magnesium were tested with flavors, as previously described (Scott and Verney, '47; Scott,

TABLE 1

Mineral composition of experimental diets

	LOW CALCIUM DIET [1]	LOW MAGNESIUM DIET	LOW POTASSIUM DIET
	%	%	%
Na	0.218	0.218	0.483
K	0.449	0.449	....
Ca	0.0047 ± 0.0005 [2]	0.610	0.610
Mg	0.046	0.010 ± 0.002 [2]	0.046
Fe	0.022	0.022	0.022
Mu	0.005	0.005	0.005
Zn	0.0005	0.0005	0.0005
Cu	0.0005	0.0005	0.0005
Co	0.00002	0.00002	0.00002
Cl	0.339	0.339	0.339
P	0.354	0.354	0.354
SO_4	0.229	0.047	0.229
CO_3	....	0.913	0.913
I	0.002	0.002	0.002

[1] Composition with respect to other constituents than calcium and magnesium was calculated from salts added to the diet.

[2] Determined by the conventional method of ashing, precipitation as oxalate and titration with permanganate.

[3] Determined spectrographically by Dr. M. E. Warga on the ash.

Verney and Morissey, '50), to discover evidences of learning. In the case of calcium carbonate the experimental method was to divide 20 weanling rats into 4 groups, each of which received one of the following diets in each of two cups during

[7] A low potassium diet was made in a manner similar to that employed in making the other diets, with the potassium dihydrogen phosphate in the usual Jones and Foster salts replaced by sodium dihydrogen phosphate and the potassium iodide by sodium iodide. Potassium-containing diets had potassium dihydrogen phosphate added, replacing the corresponding sodium salt.

the control period: (1) low calcium diet; (2) flavored [8] low calcium diet; (3) calcium carbonate [9]-containing diet; (4) flavored calcium carbonate-containing diet. Food intakes from each cup and body weight were measured daily and the cups then interchanged.

The experimental period was divided into 4 parts of 5, 5, 5 and 6 days. During the first and third parts, half the deficient and half the control animals received as choices diets (1) and (4) while the others received diets (2) and (3). During the 2nd and 4th parts, those animals previously choosing between diets (1) and (4) chose between diets (2) and (3) while those previously selecting from (2) and (3) were given (1) and (4). Oother calcium salts were tested in the same way with appropriate diets.

The effects of flavor on appetite for magnesium were determined by the same technique. Aside from different diets,[10] the experimental period was changed as follows: In the experiment with magnesium carbonate, the experimental period was divided into three parts of 5, 5 and 4 days, while in the cases of magnesium oxide and sulfate it was divided into 4 parts of 4, 4, 3 and 3 days. Between each part flavor was reversed with respect to magnesium in the usual manner.

RESULTS

Preferences for diets containing calcium carbonate over calcium-low diets were shown by both calcium-deficient and control animals, as may be seen in table 2. However, in the cases of dicalcium phosphate and calcium sulfate a distinctly greater preference for calcium-containing diets over a low calcium diet was shown by deficient animals.

There was no evidence whatever of an appetite for magnesium-containing diets in magnesium-deficient animals (table

[8] All flavored diets contained 10 p.p.m. of oil of anise.

[9] All calcium-containing diets used in the flavor experiments contained 0.66% calcium.

[10] All magnesium-containing diets used in the flavor experiments contained 0.1% magnesium.

TABLE 2

Appetite for calcium [1]

CALCIUM IN CHOICE	ANIMALS	3-WEEK CONTROL PERIOD			3-WEEK EXPERIMENTAL PERIOD				
		Body weight change	Food eaten	Calcium intake	Body weight change	Food eaten	Calcium intake	Appetite for calcium [2]	Appetite attributable to deficiency
%		*gm*	*gm*	*mg/day*	*gm*	*gm*	*mg/day*	%	%
					Calcium carbonate				
0.33	Deficient	45.9 ± 5.5	122.6 ± 12.9	0.3	62.0 ± 4.4	205.1 ± 12.4	27.3	27.7 ± 8.3	
	Control	59.3 ± 5.3	142.1 ± 14.0	22.6	65.0 ± 7.9	230.3 ± 18.3	32.0	46.1 ± 5.1	— 18.4 ± 9.7
0.66	Deficient	42.8 ± 1.8	117.2 ± 4.4	0.3	61.1 ± 2.0	215.2 ± 5.6	61.9	38.5 ± 4.8	
	Control	63.9 ± 3.1	139.4 ± 5.0	44.1	48.5 ± 4.2	203.2 ± 10.4	45.7	14.5 ± 4.7	24.0 ± 6.7
1.3	Deficient	42.9 ± 4.0	124.8 ± 8.7	0.3	60.6 ± 3.4	226.2 ± 7.6	117.3	32.8 ± 4.2	
	Control	60.3 ± 4.3	159.0 ± 9.1	98.8	54.8 ± 6.0	236.8 ± 12.6	118.1	30.7 ± 5.4	2.1 ± 6.8
2.6	Deficient	35.0 ± 1.8	111.6 ± 2.7	0.2	64.9 ± 3.6	208.2 ± 6.5	187.6	17.6 ± 8.5	
	Control	42.4 ± 3.3	125.2 ± 8.1	155.1	55.6 ± 1.5	209.1 ± 6.4	154.0	11.3 ± 11.9	6.3 ± 14.6
					Dicalcium phosphate				
0.66	Deficient	46.9 ± 2.5	131.7 ± 4.1	0.3	65.9 ± 5.3	219.5 ± 9.5	41.5	14.5 ± 5.9	
	Control	57.8 ± 3.9	144.3 ± 7.2	45.7	59.4 ± 4.8	210.6 ± 10.0	27.7	— 14.3 ± 6.3	28.5 ± 8.6
					Calcium sulfate				
0.66	Deficient	48.5 ± 2.1	130.2 ± 4.3	0.3	64.2 ± 5.4	216.9 ± 7.9	52.3	31.1 ± 7.3	
	Control	68.5 ± 3.0	147.6 ± 4.8	46.7	58.6 ± 5.6	223.5 ± 10.0	30.4	— 7.7 ± 10.1	38.8 ± 12.5

[1] All data are given in terms of the mean and the standard error of the mean.

[2] Appetite was measured in terms of per cent of total food selected from the calcium-containing diet during the experimental period minus per cent of total food eaten from the corresponding cup during the control period.

TABLE 3

Appetite for magnesium

MAGNESIUM IN CHOICE	ANIMALS	2-WEEK CONTROL PERIOD			2-WEEK EXPERIMENTAL PERIOD				
		Body weight change	Food eaten	Magnesium intake	Body weight change [1]	Food eaten	Magnesium intake	Appetite for magnesium	Appetite attributable to deficiency
%		*gm*	*gm*	*mg/day*	*gm*	*gm*	*mg/day*	%	%
					Magnesium carbonate				
0.02	Deficient	23.6 ± 6.7	68.5 ± 2.3	0.5	32.8 ± 8.1 (4)	104.4 ± 14.0	1.2	— 24.9 ± 7.3	
	Control	34.9 ± 4.0	69.3 ± 4.4	1.5	37.5 ± 2.2	112.3 ± 4.7	1.4	— 22.7 ± 10.6	— 2.2 ± 12.9
0.05	Deficient	31.9 ± 2.5	78.2 ± 4.7	0.6	53.6 ± 3.2	158.3 ± 6.5	4.6	9.0 ± 6.7	
	Control	40.3 ± 2.0	83.3 ± 3.5	3.6	49.6 ± 3.0	151.1 ± 4.6	4.8	16.1 ± 6.1	— 7.1 ± 9.1
0.10	Deficient	22.4 ± 1.9	64.5 ± 3.8	0.5	42.0 ± 5.0 (2)	119.9 ± 8.0	2.8	— 29.0 ± 5.7	
	Control	38.1 ± 2.5	81.6 ± 3.1	6.4	42.0 ± 3.8	130.7 ± 6.1	4.9	— 5.4 ± 7.8	— 23.6 ± 9.6
0.20	Deficient	23.1 ± 2.3	73.0 ± 2.8	0.5	43.1 ± 3.6 (2)	124.1 ± 6.1	5.5	— 22.2 ± 11.2	
	Control	37.6 ± 1.9	85.1 ± 2.6	12.8	34.2 ± 2.7	123.9 ± 3.5	7.6	— 15.7 ± 7.0	— 6.5 ± 13.2
					Magnesium sulfate				
0.02	Deficient	20.1 ± 1.3	62.1 ± 4.0	0.4	30.7 ± 5.0 (4)	95.6 ± 7.0	1.2	— 17.2 ± 5.2	
	Control	34.6 ± 1.7	79.2 ± 3.4	1.7	31.7 ± 1.4	113.2 ± 4.6	1.3	— 18.9 ± 8.8	1.7 ± 10.2
0.1	Deficient	32.4 ± 1.9	81.8 ± 2.7	0.6	45.0 ± 2.9	157.5 ± 3.6	4.0	— 17.9 ± 7.8	
	Control	43.0 ± 2.4	92.2 ± 5.9	7.2	43.2 ± 3.1	160.7 ± 6.7	4.7	— 25.9 ± 8.6	8.0 ± 11.6
					Magnesium oxide				
0.02	Deficient	32.8 ± 3.5	83.3 ± 6.5	0.6	42.4 ± 3.9 (3)	129.4 ± 8.0	1.5	— 11.0 ± 8.6	
	Control	42.1 ± 1.9	89.5 ± 4.1	1.9	47.3 ± 3.2	132.2 ± 7.5	2.3	25.1 ± 11.2	— 36.1 ± 14.1
0.1	Deficient	36.7 ± 2.5	80.9 ± 5.0	0.6	46.8 ± 2.9 (1)	134.0 ± 7.5	5.3	1.8 ± 7.1	
	Control	48.5 ± 2.0	93.8 ± 3.2	7.4	36.2 ± 1.8	124.7 ± 4.0	5.5	6.9 ± 5.8	— 5.1 ± 9.2

[1] Figures in parentheses represent number of animals that died during the experimental period.

TABLE 4

Appetite for potassium

POTASSIUM IN CHOICE	ANIMALS	CONTROL PERIOD			EXPERIMENTAL PERIOD				
		Body weight change	Food eaten	Potassium intake	Body weight change [1]	Food eaten	Potassium intake	Appetite for potassium	Appetite attributable to deficiency
%		*gm*	*gm*	*mg/day*	*gm*	*gm*	*mg/day*	%	%
					3-Week periods				
0.1	Deficient	11.8 ± 1.9	69.1 ± 3.4	...	31.0 ± 7.2 (3)	105.5 ± 11.9	3.1	4.4 ± 7.7	
	Control	44.6 ± 3.6	107.3 ± 6.0	5.1	42.7 ± 3.8	163.2 ± 12.2	4.4	7.6 ± 7.9	— 3.2 ± 11.0
					2-Week periods				
0.2	Deficient	5.7 ± 0.8	44.1 ± 3.5	...	28.4 ± 8.2 (2)	89.4 ± 13.0	9.2	5.2 ± 11.1	
	Control	45.0 ± 1.3	83.6 ± 1.7	11.9	53.0 ± 4.1	152.5 ± 4.9	17.0	24.5 ± 5.0	— 19.3 ± 12.2

[1] Figures in parentheses represent number of animals that died during the experimental period.

TABLE 5

Effect of flavor on appetite for calcium

ANIMALS	CONTROL PERIOD		EXPERIMENTAL PERIOD			
	Weight change	Food eaten	Weight change	Food eaten	Change in fraction eaten from cup I [1]	Fraction eaten associated with flavor [2]
	gm	*gm*	*gm*	*gm*	%	%
			Carbonate			
Deficient	45.9 ± 3.1	138.2 ± 8.0	57.6 ± 7.0	218.1 ± 14.3	27.8 ± 6.7	56.6 ± 5.5
Normal	72.4 ± 5.0	171.7 ± 9.9	45.8 ± 4.9	222.4 ± 11.6	18.8 ± 6.8	48.9 ± 4.7
			Phosphate			
Deficient	51.9 ± 2.3	145.3 ± 4.5	82.2 ± 7.8	260.8 ± 12.0	15.4 ± 3.7	61.8 ± 4.4
Normal	55.0 ± 6.1	140.6 ± 10.1	68.2 ± 6.3	228.8 ± 12.1	−2.9 ± 9.7	42.3 ± 4.1
			Sulfate			
Deficient	34.1 ± 3.8	100.8 ± 5.8	53.0 ± 5.9	199.4 ± 14.5	18.6 ± 10.9	59.9 ± 3.9
Normal	58.1 ± 4.3	134.9 ± 6.2	56.0 ± 5.1	242.9 ± 10.6	−2.9 ± 7.2	40.4 ± 3.6

[1] Control period minus experimental period. The calcium-containing diet was in cup II during the experimental period, and a positive change was therefore an indication of preference for calcium-containing food.

[2] Per cent eaten of that food, flavored or unflavored, which contained the calcium in the first part of the experimental period. A result greater than 50 was an indication of association of flavor with the beneficial effect of the calcium.

TABLE 6

Effect of flavor on appetite for magnesium

ANIMALS	CONTROL PERIOD		EXPERIMENTAL PERIOD			
	Weight change	Food eaten	Weight change	Food eaten	Change in fraction eaten from cup I [1]	Fraction eaten associated with flavor [2]
	gm	*gm*	*gm*	*gm*	%	%
Magnesium carbonate						
Deficient	31.4 ± 0.9	74.0 ± 1.5	48.8 ± 3.4	132.3 ± 6.1	— 19.4 ± 5.7	42.2 ± 4.5
Control	51.2 ± 2.3	97.1 ± 2.3	51.0 ± 5.5	152.4 ± 8.5	— 4.5 ± 6.7	45.0 ± 8.2
Magnesium sulfate						
Deficient	31.9 ± 2.6	74.2 ± 4.6	42.6 ± 3.5	127.0 ± 4.8	— 13.5 ± 7.2	55.3 ± 2.8
Control	35.6 ± 3.1	71.1 ± 5.5	35.1 ± 2.8	113.0 ± 6.6	1.2 ± 9.6	46.1 ± 4.5
Magnesium oxide						
Deficient	29.6 ± 2.2	73.9 ± 4.5	46.4 ± 4.5	142.0 ± 5.9	— 9.7 ± 10.5	39.6 ± 6.0
Control	50.7 ± 4.8	93.2 ± 7.9	56.9 ± 4.0	166.4 ± 10.7	14.8 ± 6.8	51.7 ± 3.5

[1] Control period minus experimental period. The magnesium-containing diet was in cup II during the experimental period, and a positive change was therefore an indication of preference for magnesium-containing food.

[2] Per cent eaten of that food, flavored or unflavored, which contained the magnesium salt in the first part of the experimental period. A result greater than 50 was an indication of association of flavor with the beneficial effect of magnesium salt.

3). In fact, such diets were generally avoided and in some cases avoided to the extent that animals died in typical magnesium deficiency convulsions with magnesium present in food in the cage.

No evidence was found of an appetite for potassium, as is shown in table 4.

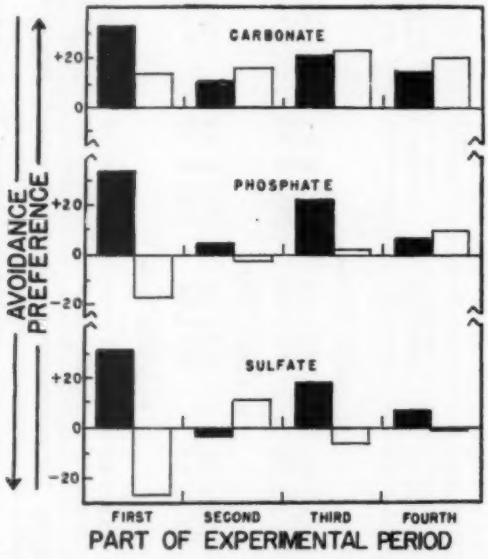

Fig. 1 Effect of change of flavor on appetite for calcium-containing diets. Flavor was changed with respect to calcium between each part of the experimental period. Preference (positive) and avoidance (negative) for calcium-containing diets were calculated as per cent of total food selected that was calcium-containing, minus 50. Open bars = control animals; closed bars = calcium-deficient animals.

In the flavor experiments with calcium, the results of which are shown in table 5 and figure 1, the eating behavior of the animals showed an obvious similarity to that exhibited in experiments on B vitamins (Scott and Verney, '47), the change with respect to preference for calcium being to one

having the characteristics of a learned appetite. The flavor experiments with magnesium, the results of which are shown in table 6 and figure 2, do not permit as simple an interpretation.

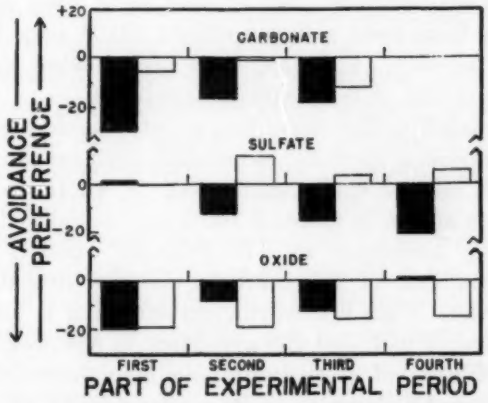

Fig. 2 Effect of change of flavor on appetite for magnesium-containing diets. Flavor was changed with respect to magnesium between each part of the experimental period. Preference (positive) and avoidance (negative) for magnesium-containing diets were calculated as per cent of total food selected that was magnesium-containing, minus 50. Open bars = control animals; closed bars = magnesium-deficient animals.

DISCUSSION

On the basis of the results of the experiments with calcium, it appears reasonable to conclude that calcium-deficient, but not normal, rats can develop a preference for calcium-containing over low calcium diets which is probably learned by the animals as a result of their experience in eating the former. That learning was involved is shown by the flavor experiments, where the rats must have learned to associate the effects of calcium with anise flavor or lack of it. The results with calcium carbonate are unusual in one respect, in that both control and deficient animals preferred diets containing this substance in comparatively large amounts. This was presumably a flavor effect unrelated to deficiency,

since both types of animals showed it, but the reason for the preference for diets containing as much as 3 to 6% calcium carbonate is not obvious.

The avoidance of magnesium found here appears to have 4 possible explanations: (1) it may have been accidental; (2) it may have been a flavor effect; (3) it may have been related to toxicity; (4) it may have been related to a feeling of well-being and thus a learned avoidance.

The probability that there is a greater avoidance of magnesium-containing diets by magnesium-deficient animals than by controls can be easily calculated. If all the magnesium experiments shown in tables 3 and 5 are considered together, the average avoidance by 110 deficient animals is 8.67% greater than that of 110 controls, and the probability that this difference is not due to random sampling is 0.989. Thus the odds are 90 to 1 that the avoidance is not accidental.

The avoidance of diets containing magnesium sulfate appears reasonable because of the bitter taste of this salt; but both magnesium oxide and carbonate, being insoluble, are as tasteless as chalk, yet there was in some cases evidence of avoidance of the latter two by control animals. In general, in experiments with mineral appetites, control animals often show an erratic preference for or avoidance of a particular salt in the diet, quite unlike the uniformity of behavior they exhibit in B vitamin experiments. This produces a complication, in that if a strong preference is present — based, it may be assumed, on flavor — a learned preference could be easily masked. This appears to have been the case with calcium carbonate above, since although deficient animals had no greater preference for this substance than the non-deficient ones in 3 of 4 simple choice experiments, the calcium carbonate flavor data are very typical of a learned appetite. Similarly, a taste avoidance could conceivably mask a learned avoidance. If the avoidance of magnesium were solely a flavor effect one would not expect a greater avoidance by deficient animals, except in the very unlikely event that magnesium deficiency

caused a specific change in taste receptivity for very small amounts of magnesium compounds. If the avoidance is a flavor effect, its basis is diametrically opposed to the explanation given by Richter ('39) of the appetite for sodium chloride. This author concluded that increased need for salt caused a change in the taste mechanism such that a higher salt intake resulted. In the case of magnesium, any postulated change in the taste mechanism would result in a lower intake of magnesium. That magnesium in the diet in the concentrations used is not toxic is obvious, so that the animals were not avoiding it for that reason.

The most plausible explanation of these results is that the avoidance of magnesium is learned. It is well-known that a feeling of well-being is not always associated with the best possible physical status nor the ultimate welfare of an individual. Examples that may be cited are those of alcoholism, narcotism, and the euphoria that may occur in high altitude anoxia. Magnesium-deficient animals are nervous and highly excitable and likely to go into convulsions on slight stimulation, while magnesium is a nervous system depressant. It is possible that the highly excitable stage of magnesium deficiency is pleasurable to the rat, and that he learns to avoid a diet that represses this pleasurable state. The experiments on the effect of flavor on appetite for magnesium did not give unequivocal results. There is a tendency toward alternation of avoidance with change in flavor in the cases of carbonate and oxide, with none in the case of sulfate, which has its own distinctive flavor. These results, while by no means conclusive, are consistent with the conclusion that a poorly defined avoidance of magnesium may be developed in magnesium-deficient rats. This avoidance is similar in some respects to the learned appetites previously found for B vitamins.

In this paper, the last of a series on appetites for specific dietary essentials, the results of all the controlled experi-

ments of this type can be summarized. This is done in table 7. It will be noted that specific appetites for 12 different essentials have been sought, and that of these, 5 appetites were demonstrated, two gave doubtful results, and in 5 cases no appetites could be shown. These results indicate marked limitations in the reliability of appetites as a means of attaining good nutrition.

TABLE 7

Appetites of the rat in relation to needs

DIETARY ESSENTIAL	APPETITE DEMONSTRATED	TYPE OF APPETITE	REFERENCE
Thiamine	Yes	Learned	Harris et al. ('33)
	Yes	Learned	Scott and Quint ('46a)
Riboflavin	Yes	Learned	Scott and Quint ('46a)
Pyridoxine	Yes	Learned	Scott and Quint ('46a)
Pantothenate	Doubtful	Learned	Scott and Quint ('46a)
Vitamin A	No		Harris et al. ('33)
Vitamin D	No		Harris et al. ('33)
	No		Young and Wittenborn ('40)
Protein	No		Harris et al. ('33)
	No		Scott and Quint ('46b)
Sodium	Yes	Doubtful	Richter ('36)
	Yes		Mark ('42)
	Yes		Clark and Clausen ('43)
	Doubtful	Doubtful	Scott et al. ('50)
Chloride	Doubtful		Scott et al. ('50)
Potassium	No		Present paper
Calcium	Yes		Richter and Eckert ('37)
	Yes	Learned	Present paper
Magnesium	No	Avoidance	Present paper

SUMMARY

Calcium-deficient rats, but not control animals, showed an appetite for diets containing this element. Magnesium-deficient rats, however, tended to avoid diets containing magnesium to a greater extent than did control animals. The appetite for calcium was similar to the learned appetites for B vitamins, and the magnesium avoidance had some char-

acteristics in common with learned appetites. No appetite for diets containing potassium was found in potassium-deficient rats.

LITERATURE CITED

CLARK, W. G., AND D. F. CLAUSEN 1943 Dietary "self-selection" and appetite of untreated and treated adrenalectomized rats. Am. J. Physiol., *139:* 70.

HARRIS, L. J., J. CLAY, F. J. HARGREAVES AND A. WARD 1933 Appetite and choice of diet. The ability of the vitamin B deficient rat to discriminate between diets containing and lacking the vitamin. Proc. Roy. Soc., *113B:* 161.

JONES, J. H., AND C. FOSTER 1942 A salt mixture for use with basal diets either high or low in phosphorus. J. Nutrition, *24:* 245.

MARK, J. 1942 Inactivation of desoxycorticosterone acetate. Endocrinology, *31:* 582.

RICHTER, C. P. 1936 Increased salt appetite in adrenalectomized rats. Am. J. Physiol., *115:* 155.

———— 1939 Salt taste thresholds of normal and adrenalectomized rats. Endocrinology, *24:* 367.

RICHTER, C. P., AND J. F. ECKERT 1937 Increased calcium appetite of parathyroidectomized rats. Ibid., *21:* 50.

SCOTT, E. M., AND E. QUINT 1946a Self selection of diet. III. Appetites for B vitamins. J. Nutrition, *32:* 285.

———— 1946b Self selection of diet. IV. Appetite for protein. Ibid., *32:* 293.

SCOTT, E. M., AND E. L. VERNEY 1947 Self selection of diet. VI. The nature of appetites for B vitamins. Ibid., *34:* 471.

SCOTT, E. M., E. L. VERNEY AND P. MORISSEY 1950 Self selection of diet. X. Appetites for sodium, chloride, and sodium chloride. Ibid., *41:* 173.

YOUNG, P. T., AND J. R. WITTENBORN 1940 Food preferences of normal and rachitic rats. J. Comp. Psychol., *30:* 261.

CALCIUM PHOSPHATE DEPOSITS IN GUINEA PIGS AND THE PHOSPHORUS CONTENT OF THE DIET [1]

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FOUR FIGURES

(Received for publication December 16, 1949)

The literature on the stiff-wrist syndrome in guinea pigs is voluminous, and only the papers that are pertinent to our own observations will be reviewed at this time. Wulzen and Bahrs ('41) reported that guinea pigs on a diet consisting chiefly of skim milk developed a characteristic syndrome which began with stiffness of the wrists. In advanced cases calcium phosphate deposits were found throughout the body. Van Wagtendonk, Freed and Ballou ('44) found that when animals had consumed the deficient diet for over a year the inorganic phosphorus in the blood plasma reached a concentration twice as high as was normal. Van Wagtendonk and Wulzen ('43, '46) isolated fractions of raw cream and of cane juice which in exceedingly minute dosage cured the wrist stiffness syndrome and reduced the inorganic phosphorus of the blood to normal levels. Oleson, Van Donk, Bernstein, Dorfman and SubbaRow ('47) and Petering, Stubberfield and Delor ('48) reported that a large number of steroids have antistiffness activity. Ergostanyl acetate and α-ergostanyl acetate were the most active.

The effectiveness of curative agents has been questioned, and Christensen, Naff, Cheldelin and Wulzen ('48) reported

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that the soreness test of van Wagtendonk and Wulzen ('43) is unreliable for a quantitative assay. Dasler and Bauer ('49) were not convinced that the syndromes described by Petering, Stubberfield and Delor ('48) and by van Wagtendonk and Wulzen ('43) were identical, and Smith, Williams, Bauer and Maynard ('49) came to about the same conclusion. Smith et al. ('49) observed wrist stiffness and tissue calcification on a variety of diets though they, as did most other workers, found the skim milk diet of van Wagtendonk and Wulzen unsatisfactory, due to a high mortality rate and the inconsistent appearance of the symptoms. They agreed with Dasler and Bauer that ergostanyl acetate had no protective activity.

So far as we are aware, no one has suggested as yet that mineral elements of the diet may be implicated in the wrist stiffness syndrome, but observations on other animals indicate that these constituents may be a determining factor. There is a considerable body of evidence that maladjustments in the intake of calcium and phosphorus by rats may result in abnormal deposition of calcium phosphate in the tissues (Stephens and Barr, '33; Barnes, '42; Saxton and Ellis, '41). According to Kintner and Holt ('32), a ration that supplies liberal amounts of phosphorus, with a low Ca:P ratio, produces serious abnormalities in the bones of horses. The present authors have observed calcium phosphate deposits in guinea pigs and the object of this paper is to describe our investigations of that abnormality. A summary of our observations appeared some time ago (Hogan and Regan, '46).

EXPERIMENTAL

The experimental procedure employed was practically the same as that used by Hogan and Hamilton ('42) except that most of the animals were weaned and started on the experimental diets at an earlier age. We prefer animals not over one week old, at weights between 150 and 175 gm. The investigations of Hogan and Hamilton were interrupted and when resumed by Hogan and Regan ('46) the earlier results could not be duplicated. In the meantime the salt mixture no. 5199,

used by Hogan and Hamilton, had been replaced by no. 5 (Richardson and Hogan, '46), and it soon developed that this change was the cause of the difficulty. Other changes in the diets were made before the importance of the salt mixture was discovered, but they were of no significance for our purpose

TABLE 1

Composition of rations

RATION NO.	711	713
	%	%
Casein	20	20
Sucrose	36	35
Cellu flour	15	15
Salts (5199)	4	
Salts (5)		5
Lard [1]	10	10
Dried brewers' yeast	15	15

[1] Added fat-soluble vitamins per 100 gm:

Vitamin A	2,000 I.U.	Vitamin E	2.5 mg
Vitamin D	283 I.U.	Vitamin K_3	2.5 mg

	%	%
Calcium	0.8	0.8
Phosphorus	0.5	0.9

Stock colony diet no. 16

Ground wheat	25	Dried yeast	15
Wheat germ	6	Fish liver oil	1
Wheat shorts	10	Steamed bone meal	2
Alfalfa leaf meal	40	Sodium chloride	1

Manganous sulphate, hydrated 0.025

Protein	21.6
Calcium	1.23
Phosphorus	0.85

Each animal was given every other day a gelatin capsule which contained 20 mg of ascorbic acid.

and will not be described. Typical diets containing these two salt mixtures are shown in table 1. The stock colony diet now in use is also described, as it has been quite satisfactory during the stages of growth, gestation and lactation.

After the importance of the salt mixture was recognized the diets were modified at times to determine whether the rate of growth was affected by the type of casein, cellulose or other

carbohydrates in the diet, or by the addition of various members of the vitamin B complex. However, these changes were of no significance for our purpose and will therefore not be described. The results obtained with salt mixture no. 5199 are all ascribed to diet 711, those with mixture 5 to diet 713. The animals that consumed salts 5 grew more slowly, the mortality rate was higher, and the females did not reproduce. Specific abnormal symptoms were not noticed until the animals were 5 months old or older, when inflammatory spots began to appear on the pads of the feet. These begin as small white pinheads which later on expand and become necrotic. Their appearance suggests a focus of infection but when the contents are examined under the microscope it is seen that the spots are due to the accumulation of minute particles which are not bacteria. Chemical analysis showed they are made up chiefly, or entirely, of calcium phosphate. These particles are so small that they are beyond the resolving power of a petrographic microscope. A sample was sent to the x-ray laboratory for examination and the operator [2] reported that the sample was examined by the Debye-Scherrer powder method and "the X-ray pattern produced indicated the presence of crystalline material."

It is assumed that the calcium phosphate deposits are of the same type as those described by Wulzen and Bahrs ('41) and by Smith, Williams, Bauer and Maynard ('49). They may first form in almost any portion of the body, on the top or side of the toes, on the elbows, ribs, and on the spinal column. If near a bone they become attached to it and resemble a bony overgrowth. They may appear in the connective tissue between the skin and muscle layers, and large collections have been observed on the stomach and on the duodeno-jejunal flexure. Microscopic calcium phosphate crystals are abundant between the muscle fibers of affected animals. A portion of

[1] Mr. J. H. Affleck examined the sample and prepared the report.

[2] Kindly prepared by M. P. Neal, M.D., Professor of Pathology in the School of Medicine, University of Missouri. Dr. Neal also prepared the slides for figures 3 and 4 and wrote the legends that accompany them.

the University pathologist's report [3] on a typical animal reads as follows:

"Histopathology: Multiple sections of the lesions show three rather basic changes:

1. "Degenerative change of musculature, with swelling of muscle fibers, some fragmentation, loss of striations, and change in staining reaction from acidophilic to basophilic.

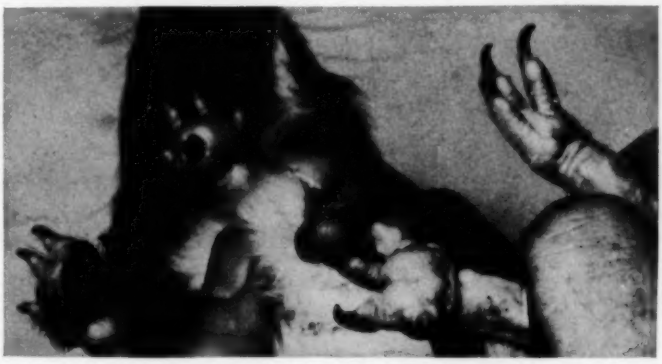

Fig. 1 Guinea pig 598. Age, 306 days. Weight, 295 gm. Lesions of varying severity on pads of all 4 feet.

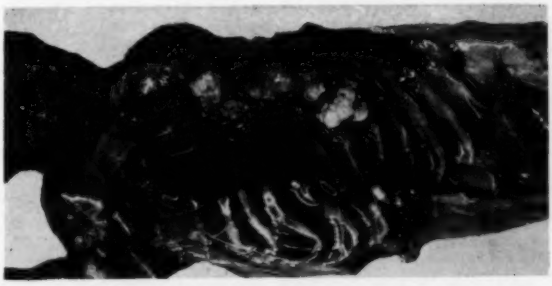

Fig. 2 Guinea pig 558. Age, 617 days. Weight, 432 gm. Masses of calcium phosphate crystals near rib attachment to spinal column.

2. "Deposition of mineral salts in soft tissues, particularly of muscles, muscle fascial planes, and outside the periosteum of bones.
3. "A chronic inflammatory reaction, especially marked around the areas of mineral deposits, with the presence of foreign body giant cells, endothelial leucocytes, and fibroblasts."

Illustrative photographs of the lesions are reproduced in figures 1, 2, 3 and 4.

When the mineral deposits were first observed all the experimental animals were consuming diet 713, or one practically

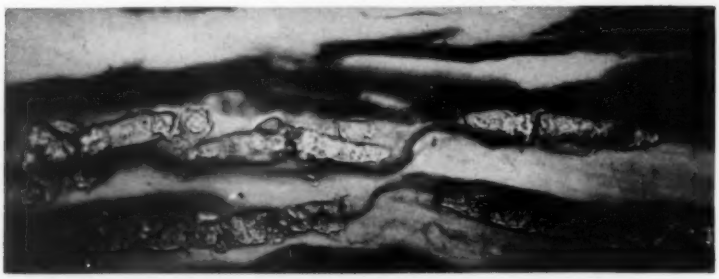

Fig. 3 Guinea pig 24. Age, 338 days. Weight, 443 gm. Skeletal muscle: Partial loss of striations; basophilic staining and parallel deposits of crystalline material along the connective tissue stroma.

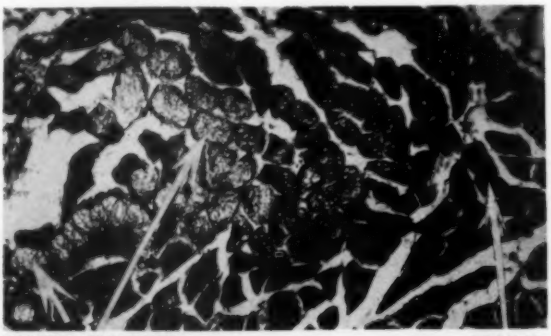

Fig. 4 Guinea pig 7. Age, 196 days. Weight, 408 gm. Skeletal muscle, cross section: Degenerative change with basophilism of cytoplasm; swelling of fibers and granular crystal deposits.

identical with it. In order to test the possibility that the salt mixture was implicated in the abnormality, the guinea pigs which were free from detectable deposits were divided into two groups. One group of 10 was continued on salts 5, and 9

of them developed mineral deposits in an average of 237 days. Another group of 11 was changed to salts 5199, and 6 developed mineral deposits in an average of 313 days. In addition to the 10 guinea pigs just mentioned, which received salts 5 continuously, 22 more were started later, making 32 in all which continuously received this mineral mixture. Of these 32 animals, 28, or 87.5%, developed mineral deposits within a period of 150 to 623 days. There were 37 animals which received salts 5199 continuously and of this number three developed mineral

TABLE 2

The relation between the phosphorus content of the diet and the incidence of calcium phosphate deposits

AGE INTERVAL, DAYS		180–200	201–250	251–300	301–400	401–500	501 +	TOTALS
Salts	a [1]	2	5	4	10	7	6	34
5199	b	0	0	0	0	3	0	3
Salts	a	0	1	2	0	0	1	4
5	b	3	4	2	10	4	5	28

[1] The numerals in the various columns show the number of animals that died, or were discontinued on experiment for any other reason, during the age interval indicated. In some cases deposits had been observed at an earlier interval.

Line a: Calcium phosphate deposits were not observed.

Line b: Calcium phosphate deposits were observed.

For example, 5 guinea pigs on salts 5 died or were discontinued during the age interval 201 to 250 days. Calcium phosphate deposits were visible in 4 animals and could not be detected in the 5th.

deposits, at the ages of 411, 412 and 479 days. This is a low incidence but it is strong evidence that ration 711, which contains salts 5199, is slightly unsatisfactory in some respect. A summary of our data is shown in table 2.

Additional evidence that salt mixture 5 is unsatisfactory is provided by the mortality records. There were 19 animals on this mixture that died a natural death and 12, or 63%, died before they were 450 days old. There were 14 animals on mixture 5199 that died a natural death and 4, or less than 29%, died at an age less than 450 days. The colony diet contains

practically the same percentage of phosphorus as does diet 713 but it contains about 50% more calcium. It is evident, therefore, that the total quantity of phosphorus in diet 713 does not explain why the animals that consume it develop deposits of calcium phosphate. It may be that within limits the Ca : P ratio is of decisive importance, though an organic factor may be involved.

The effect of the salt mixture on the rate of growth of the animals included in table 2 is shown in table 3. The guinea pigs that consumed diet 711 did not grow at the maximum rate but they grew more rapidly than did those that consumed diet 713.

TABLE 3

Effect of salt mixture on the average daily weight gains of guinea pigs

AGE INTERVAL	SALT MIXTURE			
	5199		5	
	Males [1] (15)	Females (22)	Males (17)	Females (15)
	gm	*gm*	*gm*	*gm*
0–6 weeks	5.6	4.6	5.0	3.8
6–12 weeks	4.7	3.3	2.7	2.6

[1] The number of animals is shown in parentheses.

Several types of tissue were analyzed for total mineral content. The quantity of ash in the muscle and stomach was much higher than the normal amount. The quantity in the heart, liver, and lungs was only about 25% higher than is normal. The analyses are summarized in table 4.

Soon after the calcium phosphate deposits were first observed we began examining the animals for the wrist soreness syndrome, by the method described by van Wagtendonk and Wulzen ('43). There was no difficulty in confirming their report in all essential respects. According to our observations, the disease can be divided roughly into three stages: (1) Pain in the wrist when the joint is manipulated; (2) the wrist, elbow, or both become stiff; (3) visible, or palpable, deposits of calcium phosphate. The first symptom is not uncommon in

animals that consume either diet 711 or the colony diet, and a small proportion that develop the first symptom also develop the second. Practically all animals on diet 713 develop, in order, all three symptoms. One would assume from our data that all three symptoms indicate varying degrees of severity of the same disease, but we have no experimental evidence of identity. Raw cream was tested on a few animals as a curative agent (van Wagtendonk and Wulzen, '46) with entirely negative results. After the experimental work described in this report had been completed we tried cane molasses, which van Wagtendonk and Wulzen ('46) had found to be active, but in

TABLE 4

Percentage of ash in moisture- and fat-free tissues

DIET	NO. OF SAMPLES	MUSCLE	STOMACH	HEART	LIVER	LUNGS	KIDNEYS	FEMUR
713	9	8.9	15.3	6.3	6.6	6.8	6.5 (6)	63.3
711	9	5.7 (8)[1]	6.3 (8)	5.7	5.6	6.6 (8)	..	64.7
16	5	5.8	6.1	5.2	4.7	6.1	6.7 (3)	58.3

[1] When some of the samples were not analyzed, the actual number represented in the average is shown in parentheses.

our hands it was no more effective than was cream. It should be stated, however, that while the sore wrist stage undoubtedly occurs, it is an unreliable method of diagnosis. The response of animals to the test from day to day is quite erratic. Evidently Christensen, Naff, Cheldelin and Wulzen ('48) had the same experience.

We obtained limited evidence that the deposition of calcium phosphate deposits is a reversible process. One female that had large deposits and was in wretched physical condition was changed to the colony diet at the age of 200 days. At the age of 292 days the deposits were not detectable and the animal was in excellent condition. She bore a litter of two at the age of 618 days, and reared it successfully.

SUMMARY

Approximately 90% of the guinea pigs that consumed a synthetic diet which contained 0.8% of calcium and 0.9% of phosphorus developed visible deposits of calcium phosphate. If the total amount of phosphorus was reduced to 0.5%, the incidence of the deposits was less than 10%. The Ca:P ratio may be as important as the absolute amounts.

When animals consumed the high-phosphorus diet they grew more slowly and the survival period was shortened. The soft tissues contained an abnormally high percentage of mineral constituents.

Under the experimental conditions described wrist soreness and wrist stiffness invariably preceded the appearance of visible calcium phosphate deposits.

LITERATURE CITED

BARNES, LEROY L. 1942 The deposition of calcium in the hearts and kidneys of rats in relation to age, source of calcium, exercise and diet. Am. J. Path., *18:* 41.

CHRISTENSEN, B. E., M. B. NAFF, V. H. CHELDELIN AND ROSALIND WULZEN 1948 A study of the assay method for the guinea pig antistiffness factor. J. Biol. Chem., *175:* 275.

DASLER, W., AND C. D. BAUER 1949 Assay for antistiffness activity with guinea pigs depleted on solid rations. Proc. Soc. Exp. Biol. Med., *70:* 134.

HOGAN, A. G., AND J. W. HAMILTON 1942 Adequacy of simplified diets for guinea pigs and rabbits. J. Nutrition, *23:* 533.

HOGAN, A. G., AND W. O. REGAN 1946 Diet and calcium phosphate deposits in guinea pigs. Proc. Fed. Am. Soc. Exp. Biol., *5:* 138.

KINTNER, J. H., AND R. L. HOLT 1932 Equine osteomalacia. Philippine J. Sci., *49:* 1.

OLESON, J. J., EVELYN C. VAN DONK, S. BERNSTEIN, L. DORFMAN AND Y. SUBBAROW 1947 Steroids and the stiffness syndrome in guinea pigs. J. Biol. Chem., *171:* 1.

PETERING, H. G., L. STUBBERFIELD AND R. A. DELOR 1948 Studies on the guinea pig factor of Wulzen and van Wagtendonk. Arch. Biochem., *18:* 487.

RICHARDSON, L. R., AND A. G. HOGAN 1946 Diet of mother and hydrocephalus in infant rats. J. Nutrition, *32:* 459.

SAXTON, J. A., JR., AND G. H. ELLIS 1941 Effects of long-continued ingestion of sodium phosphate upon the parathyroids, kidneys and bones of mature rats. Am. J. Pathol., *17:* 590.

SMITH, S. E., M. A. WILLIAMS, A. C. BAUER AND L. A. MAYNARD 1949 The wrist stiffness syndrome in guinea pigs. J. Nutrition, *38:* 87.

STEPHENS, D. J., AND D. P. BARR 1933 Influence of acid and phosphate on metastatic calcification. Proc. Soc. Exp. Biol. Med., *30:* 920.

VAN WAGTENDONK, W. J., ANNA M. FREED AND C. E. BALLOU 1944 A dietary factor essential for guinea pigs. V. Phosphorus and calcium content of the blood and muscle during deficiency. Arch. Biochem., *5:* 329.

VAN WAGTENDONK, W. J., AND ROSALIND WULZEN 1943 A dietary factor essential for guinea pigs. I. Isolation from raw cream. Ibid., *1:* 373.

———— 1946 A dietary factor essential for guinea pigs. VIII. The isolation of the antistiffness factor from cane juice. J. Biol. Chem., *164:* 597.

WULZEN, ROSALIND, AND ALICE M. BAHRS 1941 Effects of milk diets on guinea pigs. Am. J. Physiol., Proc., *133:* 500.

LYSINE DEFICIENCY IN RATS

I. STUDIES WITH ZEIN DIETS [1]

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FOUR FIGURES

(Received for publication December 21, 1949)

INTRODUCTION

Although extensive evidence in the literature (i.e., Osborne and Mendel, '14, '15; Winternitz, '42; Harris et al., '43) indicates it is easy to obtain the picture of lysine deficiency and that rats are readily maintained for long periods of time as stunted animals, little information is at hand showing a specific role for lysine either in the synthesis of the growth hormone or in its activity. It was the original purpose of this study, therefore, to produce an absolute and uncomplicated lysine deficiency in order to gain information on the above-mentioned points.

Because the protein gliadin contains some lysine, zein was selected for the production of lysine deficiency since it is completely deficient in this amino acid. It soon became evident, however, that animals fed a supplemented zein diet grew at a rate far below the optimum of rats fed a casein ration. This was further confirmation of the observations of other workers (Geiger and Hagerty, '49; Borchers et al., '42; Harris et al., '43).

[1] The authors are indebted to the National Vitamin Foundation, New York, New York, and to the James Hudson Brown Memorial Fund of the Yale University School of Medicine for grants in support of this work.

Because of these results, our attention was diverted from the original objective to an attempt to clarify the reasons for growth failure on the completely supplemented zein diet. An account of this study follows.

TABLE 1

Percentage composition of diets

COMPONENT	DIETS			
	A	B	C	B-4
	%	%	%	%
Dextrin [1]	55.7	49.2	50.2	24.2
Casein [2]	20.0			
Zein		25.0	25.0	
Vitaminized casein [3]	5.0			
Vitaminized dextrin [3]		5.0	5.0	5.0
Lard [4]	10.0	10.0	10.0	10.0
Corn oil [5]	5.0	5.0	5.0	5.0
Salts IV [6]	4.0	4.0	4.0	4.0
L-cystine	0.2	0.2	0.2	0.2
Choline HCl	0.1	0.1	0.1	0.1
L-lysine		1.0		1.0
DL-tryptophan		0.3	0.3	0.3
DL-histidine		0.2	0.2	0.2
Zein hydrolysate [7] (50% zein, 50% dextrin)				50.0

[1] Amijel. Corn Products Refining Company, New York, New York.

[2] Labco devitaminized casein (Borden).

[3] Prepared to contain, per kilogram casein or dextrin, 100 mg each of B_1, B_2, B_6 and 2-me-naphthoquinone (Menadione); 1 gm each of niacin and pantothenic acid; 10 mg each of biotin and folic acid; 2 gm each of inositol and para-aminobenzoic acid (PABA). An oral supplement of fat-soluble vitamins was given once weekly (0.1 ml per animal) and prepared as follows: 50 ml halibut liver oil plain (Abbott), 18.0 gm viosterol, 12.5 gm α-tocopherol, brought up to 200 ml with Mazola corn oil. Each milliliter contains approximately 15,000 U.S.P. units vitamin A, 1,050 U.S.P. units D_3 (Drisdol), and 50 mg vitamin E.

[4] Swift 'nin.

[5] Mazola.

[6] See Hegsted et al. ('41). Hegsted's salts preparation was modified to contain cobalt ($CoCl_2 \cdot 6H_2O$, 0.6 gm).

[7] Described in text.

EXPERIMENTAL

Diets

Three basal diets, A, B, and C, were used, as described in table 1. Individual amino acids, and amino acids in groups of two and three, were added to give the levels in the diet suggested by Rose ('37, '38; table 2).

A zein hydrolysate was prepared as follows: (a) *Pepsin digestion.* After adjustment of the pH to 1.5 to 2.0 with HCl,

TABLE 2

Levels of essential amino acids in zein, in diet B, and in supplements to diet B

AMINO ACID	IN ZEIN [1]	IN DIET B [2]	REQUIREMENT FOR GROWTH [3]
	%	%	(% OF DIET)
Lysine	0.0	1.00	1.0
Tryptophan	0.1	0.32	0.2
Histidine	0.9 ± 0.2	0.42 ± 0.05	0.4
Phenylalanine	6.4 ± 0.7	1.60 ± 0.18	0.7
Leucine	23.7 ± 2.1	5.92 ± 0.52	0.8
Isoleucine	4.3 ± 0.4	1.07 ± 0.10	0.5
Threonine	2.4	0.60	0.5
Methionine	2.4	0.60	0.6
Cystine	0.8 ± 0.1	0.40 ± 0.02	
Valine	2.4 ± 0.9	0.60 ± 0.22	0.7
Arginine	1.6 ± 0.2	0.40 ± 0.05	0.2

[1] Block and Bolling ('45), p. 304.

[2] Calculated from figures in column one, plus supplementation as described in table 1.

[3] Rose ('37).

3.0 gm of pepsin were added to 500 gm of zein in 3 l of water, and the mixture was stirred vigorously and continuously during the digestion period. Digestion was carried on at 37°C. for 48 hours. (b) *Pancreatin digestion.* After completion of the peptic digestion, the pH was adjusted to 6.8 to 7.0 with NaOH, 24 gm of $NaHCO_3$ were added, and the pH was then stabilized at 8.0. Three grams of pancreatin were added and digestion was carried on as with the pepsin, again with constant stirring. (c) Five hundred grams

of dextrin [2] were added to the digest, which was then dried at 60°C. The dried mixture was ground and mixed with the other ingredients of diet B-4 (table 1).

Animals

Sprague-Dawley weanling male and female rats of the same age were used throughout the experiment. They were housed in individual, screen-bottom cages in a room with controlled temperature (25 to 26°C.) and relative humidity (50 to 55%). The number of animals used in each phase of the experiment is indicated in table 3.

Supplementation

Of the workers who have attempted to supplement zein diets with various amino acids in an effort to improve observed growth rates, none reported satisfactory results (Geiger, '47; Geiger and Hagerty, '49; Borchers et al., '42; Harris et al., '43). It was felt desirable, nevertheless, to continue to explore this approach. In general the technique adopted was to add amino acids individually or in groups of two or three to diet B at levels suggested by Rose ('37, '38).

In order to eliminate the possibility that the poor growth obtained with zein (to which tryptophan, lysine, histidine, and cystine had been added, i.e., basal diet B) was due to a "toxic" factor present in zein, a complete amino acid supplement was given to two animals on diet B. For much the same purpose, 5% of vitamin-free casein [3] was added to diet B. The various supplements, and the average weight gain per animal per day, are reported in table 3. In order to evaluate the possible role of some of the newer growth factors, certain animals on diet B received supplements of liver extract,

[2] The dextrin used was Amijel, obtained from the Corn Products Refining Company, New York, New York.

[3] Labco vitamin-free casein was obtained from the Borden Company, New York, New York.

TABLE 3

Growth responses to diet supplementation

DIET	SUPPLEMENT [1]	NO. OF ANIMALS	NO. OF DAYS	AVERAGE GAIN/ DAY/ANIMAL	
				gm/day	
A	None	8	40	4.1	
A	None	4	67	4.7	
A	None	3	40	5.8	
B	None	28	87	0.9	
B	Complete amino acid	2	6	4.6	
B	5% casein	4	26	4.4	
B	5% casein	3	42	5.8	
B	Threonine	2	6	1.1	
B	Arginine	2	5	1.7	
B	Isoleucine	2	4	1.3	
B	Phenylalanine	2	6	1.1	
B	Valine	4	13	2.8	
B	Leucine	2	7	1.2	
B	Glycine [2]	4	18	0.8	
B	Lysine [3]	5	4	1.5	
B	Arginine, threonine	2	16	2.6	
B	Arginine, isoleucine	2	5	2.2	
B	Arginine, valine	2	6	2.6	
B	Threonine, valine	4	16	1.4	
B	Threonine, isoleucine	2	5	2.1	
B	Arginine, threonine, isoleucine	2	6	1.4	
B	Arginine, threonine, phenylalanine	2	7	0.4	
B	Arginine, threonine, histidine	2	7	1.6	
B	Arginine, threonine, leucine	2	7	1.2	
B	Arginine, threonine, valine	2	7	4.1	3.2 ave.
B	Arginine, threonine, valine	8	29	2.6	
B	Arginine, threonine, valine	6	42	2.9	
B-4	None	3	27	0.4	
B-4	Arginine, threonine, valine	5	23	2.5	
B-4	Arginine, threonine, valine + growth hormone [4]	3	7	2.7	
B	B_{12} (2 μg/day) [5]	3	8	0.3	
B	Liver extract [6]	4	4	1.1	
B	APF [7]	4	4	1.7	

[1] Levels of amino acid supplementation in grams per 100 gm of diet, except where otherwise indicated, are as follows: DL-histidine 0.4; DL-phenylalanine 0.8; L-leucine 0.9; DL-isoleucine 1.2; DL-threonine 1.2; DL-methionine 0.7; DL-valine, 1.6; and L-arginine 0.3.

[2] Added to the diet at a level of 1%.

[3] Fifty milligrams per milliliter of L-lysine in physiological saline; 1 ml/day was given intraperitoneally.

[4] Growth hormone, 4 mg per day for three days, given intraperitoneally.

[5] Squibb Rubramin (15 μg/ml) was used, diluted in physiological saline and given intraperitoneally at the rate of 1 ml/day.

[6] Fifteen units liver extract composite (regular, Lederle) were given at the rate of 1 ml/day orally.

[7] Animal protein factor (Lederle) was given orally at the rate of 1 ml/day.

the animal protein factor, and vitamin B_{12}, as is indicated in table 3. The growth hormone was given to animals receiving diet B-4 supplemented with arginine, threonine and valine.

RESULTS

Zein supplemented with amino acids

The rate of growth of animals fed diet B ad libitum (i.e., zein with histidine, lysine, tryptophan, and cystine added at theoretically adequate levels) was markedly suboptimum when compared with the growth rates of three groups of animals given diet A (25% casein; table 3). By contrast, the average growth rate of animals fed diet B with the addition of a supplement of all 10 "essential" amino acids compared very favorably with that of animals on diet A. Similarly, the addition of 5% casein to diet B insured a good growth response. Results with the complete amino acid supplement and the 5% casein supplement indicated that no "toxic" growth-inhibitory factor is present in zein or is released from it during digestion.

Supplementation of diet B with essential amino acids (table 3), individually and in groups of two and three, gave favorable results in a number of instances. It is recognized that the numbers of animals used in the supplementation experiments were small, but these were in the nature of exploratory trials. In no case, however, did growth rates equal those obtained with the complete amino acid supplement or with diet A. The growth results obtained, however, with valine, valine-arginine, and arginine-threonine supplementation suggested the use of a supplement containing all three of these amino acids. Such a supplement was tried with three groups of animals, with comparable results in each group. The average growth rate per animal per day was 3.2 gm, as compared with the optimum rate of 4.0 to 4.5 gm/day/animal using diet A (the casein diet).

Zein hydrolysate diet. Considerable difficulty was encountered in digesting zein *in vitro* with pancreatin and pepsin.

Growth on a diet containing the zein hydrolysate supplemented with lysine, cystine, tryptophan and histidine (diet B-4) was very poor (table 3). The addition to diet B-4 of valine, arginine, and threonine elicited a favorable growth response nearly comparable to that obtained by the addition of these three amino acids to the unhydrolyzed zein diet (diet B), but not comparable to the growth obtained with 25% casein.

The intraperitoneal administration of 4 mg of pure growth hormone [4] each day for 4 days failed to provide additional growth stimulus (see table 3).

The animals given diet B-4 exhibited marked diarrhea, which may have been due to the excess chloride in the diet resulting from the preparation of the zein hydrolysate. This may, in part, account for the incomplete growth response observed with the addition of arginine, threonine and valine to the zein hydrolysate diet.

Zein diet without added lysine

As might be expected, animals on the lysine-deficient diet, diet C, failed to grow and, in fact, showed a steady, slight decline in weight as the experiment progressed. In contrast to the observations made of animals fed diet B, symptoms of severe deficiency appeared early in the experiment, including ruffled hair, poor stance and gait, and emaciation (see fig. 1). Survival on the diet was good. Of 25 animals fed diet C, all were alive after 38 days on the diet, 20 were alive after 67 days, and 18 were alive even after 87 days. Histological studies of the liver, heart, kidneys, adrenals, and thyroid of the "lysine"-deficient animals, using a hematoxylin and eosin stain, gave no significant evidence of pathology. A possible exception was the liver, in which the normal amounts of fat seemed to be missing. A group of animals whose intake of diet B was limited to the amount eaten by the "lysine"-deficient animals grew slowly, without the appearance of the

[4] Purified growth hormone was obtained through the courtesy of Dr. A. E. Wilhelmi.

aforementioned deficiency symptoms. The growth rate of these animals pair-fed on diet B was 0.3 gm/day, appreciably less than that of animals fed diet B ad libitum (0.9 gm/day).

Four females placed on diet B (with lysine) after 24 days on diet C (without lysine) showed immediate disappearance of the characteristic deficiency symptoms. However, growth rates were still retarded, as with animals fed diet B from the beginning. The weight gains of the 4 animals averaged only 1.2 gm/day, compared once again with the optimum rate of 4.5 gm/day on diet A.

By contrast, two females placed on diet A after 30 days on diet C grew at an average rate of 3.9 gm/day, although two others placed on diet A after a longer period of 80 days on diet C showed a less favorable growth response of 2.7 gm/day. Thus the lysine-deficient diet (diet C), even after relatively long periods of feeding, does not greatly impair the animals' potential for growth when they are placed on an adequate diet (diet A).

It was further observed that 8 females placed on diet C (lacking lysine) after 67 days on diet B (with lysine) lost an average of 24 gm in 31 days, which represents an average of one-sixth of their body weights, without the appearance of the deficiency symptoms noted above. Thus the presence of lysine in the diet under these circumstances appears to be essential not only for growth but for maintenance of body weight, although the animals tested could be considered adult only from the standpoint of age since they were still below their normal size due to their long regimen (67 days) on diet B.

Miscellaneous observations

To make certain that the lack of adequate growth of the animals on diet B was not due to a possible vitamin deficiency, resulting from some destruction or loss of vitamins in the diet, the daily intake of vitamins was increased through administration of an oral supplement consisting of more than adequate amounts of the water-soluble vitamins. Average

growth during the period of supplementation was 0.3 gm/day/animal; an unsupplemented control group on diet B gained 0.5 gm/day/animal. There was therefore no significant increment in weight after supplementation with extra water-soluble vitamins.

Supplementation of diet B with vitamin B_{12}, the animal protein factor, and liver extract yielded consistently negative results in that no improvement in growth rates was noted (table 3).

To eliminate another possible variable operating among the various groups of animals, the data for average daily food consumption during the growth period were examined. To illustrate, in one period food consumption on diets A, B, and C was 11.1, 4.8, and 3.1 gm/day, respectively. When these food consumption data are related to the weights of the animals on the above diets, these seemingly large differences vanish, since food intakes were 0.09 gm, 0.09 gm, and 0.08 gm per gram of body weight on diets A, B, and C, respectively. The possibility that an appetite factor may have influenced the growth of the deficient animals appears therefore to be ruled out. This point is given further emphasis in the results obtained with the pair-fed animals.

The onset of the estrous cycle was determined in all females, and was found to be correlated with body weight in that all females exhibited estrus when weights of 120 to 140 gm were reached, regardless of the age of the animal and the diet used. The animals on diet C, of course, never reached adequate weights for estrus to appear, but the females whose growth was delayed by the feeding of diet C nevertheless had estrous cycles upon attaining adequate body weights on a non-deficient diet (diet A), despite the fact that they were then 80 to 140 days old.

DISCUSSION

Osborne and Mendel ('14, '15) found that zein diets did not support either the growth or maintenance of the rat; however, supplementation with 3% lysine and 3% trypto-

phan resulted in growth rates equal to those of animals on casein diets. Hart et al. ('18), on the other hand, reported inadequate growth, though some gains in weight, on diets containing zein, 0.54% tryptophan, and 1% lysine. The results in the present study with zein diets seem to indicate that a very poor rate of growth is obtained with 1% lysine given in the diet, and even with higher percentages of lysine given intraperitoneally (table 3). These results corroborate those obtained by Geiger and Hagerty ('49), who used zein supplemented with 0.25% tryptophan, 1.2% lysine, 0.5% histidine, and 1% arginine. Similarly, Harris et al. ('43) obtained increases in weight of about 1 gm/day with a zein diet supplemented with lysine (40 mg/day) and tryptophan (10 mg/day), as compared with our observed rate (table 3) of 0.9 gm/day using diet B. It appears well substantiated, therefore, that zein, even when supplemented with amino acids that it does not contain, will still not support the maximum growth of young rats. It should be pointed out, however, that Osborne and Mendel used a control diet which produced growth rates in their animals far lower than those considered optimum today. Thus the growth rate they obtained with a supplemented zein ration might well have approximated that obtained with their casein control diets and still might have been small in terms of the increases in weight obtained today with improved rations.

Despite the poor response to a proteolytic digest of zein observed by Geiger and Hagerty ('49) and in the present study, it is not possible to agree with the above authors that the "delayed release of some amino acids during digestion . . . cannot be responsible for the failure to produce growth." In the first place, only 65% hydrolysis of the zein was obtained by Geiger and Hagerty. Secondly, they observed at the same time a good growth response to an acid digest of zein in which, presumably, 100% of the nitrogen was available to the animal as amino nitrogen.

Similarly, Albanese et al. ('49) observed that digestion of zein for 6 hours with pancreatin and trypsin released only

one-third of the amino nitrogen released from casein in a comparable digestion period. It appears, therefore, that certain of the important peptide linkages in zein resist proteolytic digestion both *in vivo* and *in vitro*.

It is interesting that low levels of casein supplementation of diet B (zein diet with lysine and tryptophan) resulted in a growth rate comparable to that obtained with the 25% casein diet (diet A). Chemical analysis (Block and Bolling, '45) shows that 5% casein added to diet B provided only 0.2% arginine, 0.2% L-threonine, and 0.35% L-valine in addition to the amounts already present in the zein. By comparison, supplementation with L-arginine, DL-threonine, and DL-valine provided levels of the L form of these amino acids in diet B of 0.3%, 0.6%, and 0.8%, respectively; yet the growth which resulted from such supplementation was below that obtained with the casein diet A. The indication is that the rate of release of essential amino acids from casein, while faster than that from zein, nevertheless approximates more closely the rat's requirements in terms of optimum physiological utilization for protein synthesis and growth than does the rapid absorption following the feeding of the free amino acids. Reference to table 2 shows that of the essental amino acids, phenylalanine, leucine, and isoleucine are present in diet B in amounts far in excess of their dietary requirements. Tryptophan, histidine, lysine, and cystine may be said to be present in adequate amounts, particularly since supplements of these amino acids were added to the zein in the basal diet. Valine, threonine, and arginine, however, may be considered to be present in adequate amounts in zein only if chemical analysis of the protein is used as the sole criterion for determining its nutritive value. Unfortunately, chemical analyses do not take cognizance of such factors as rates of hydrolysis under physiological conditions, rates of absorption, and the importance of having all essential amino acids in the diet available to the animal for protein synthesis at approximately the same time (cf. discussions of amino acid imbalance by Elvehjem and Krehl, '47; and Geiger, '47). In terms of the present

discussion, a more normal complement of essential amino acids from the physiological point of view was obtained in this experiment when arginine, threonine, and valine were added as pure amino acids.

The fact that the improved growth rate using arginine, threonine and valine was nevertheless lower than that obtained with 25% casein may be explained in part by the observation that the rate of release of free amino nitrogen from zein in proteolytic digestion is very slow, and that absorption of the pure amino acids added to the zein ration was probably more rapid than absorption following the release of essential amino acids from the zein during its digestion. Thus an "amino acid imbalance" could have resulted.

The possibility that a toxic factor is present in the zein must be discarded in view of the evidence that animals fed 25% zein will nevertheless grow rapidly when a complete supplement of the "essential" amino acids is available to them. The current study has shown that an adequate amino acid supplement can be devised, based on the chemical analysis of the protein, but that due to the discrepancy between the chemical analysis and the physiological value of the zein, maximum growth rates cannot be obtained with this supplement. There is evidence in the literature that this discrepancy exists not only for zein but also for gelatin (Krehl et al., '46a, b), corn gluten (Albanese et al., '49) and soybean protein (Melnick et al., '46).

Whether the deficiency symptoms seen as the result of feeding lysine-deficient diet C are specific for lysine can better be determined after feeding lysine-free diets containing pure amino acids as the source of nitrogen rather than lysine-free proteins such as zein, which complicate the deficiency syndrome.

SUMMARY

1. Zein, even when supplemented to theoretically adequate levels with the essential amino acids it does not contain and fed in an otherwise adequate diet, does not promote optimum growth in young rats.

2. Experiments were undertaken in which zein was further supplemented with essential amino acids. The simultaneous addition of arginine, threonine, and valine resulted in a good, though not maximum, growth response.

3. Similarly, when threonine, valine, and arginine were added together to a proteolytic digest of zein, a fair growth response was obtained.

4. These results appear to support the hypothesis that zein is not adequately digested by the growing rat, and that consequently, even when supplemented with a variety of pure amino acids, it does not support a maximum growth response, since all the amino acids esential for protein synthesis are not made available to the animal at the same time.

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LITERATURE CITED

ALBANESE, A. A., S. I. SNYDERMAN, M. LEIN, E. M. SMETAK AND B. VESTAL 1949 The biological value of corn and wheat proteins in the male infant, with a note on the utilization of D-tryptophan. J. Nutrition, *38:* 215–224.

BLOCK, R. J., AND D. BOLLING 1945 The Amino Acid Composition of Proteins and Foods. Charles C. Thomas, Springfield, Illinois.

BORCHERS, R., J. R. TOTTER AND C. P. BERG 1942 Threonine deficiency in hydrolysates of zein prepared by autoclaving. J. Biol. Chem., *142:* 697–704.

ELVEHJEM, C. A., AND W. A. KREHL 1947 Imbalance and dietary interrelationships in nutrition. J. Am. Med. Assn., *135:* 279–287.

GEIGER, E. 1947 Experiments with delayed supplementation of incomplete amino acid mixtures. J. Nutrition, *34:* 97–111.

GEIGER, E., AND E. B. HAGERTY 1949 Growth of rats on hydrolyzed zein. Arch. Biochem., *21:* 239–241.

HARRIS, H. A., A. NEUBERGER AND F. SANGER 1943 Lysine deficiency in young rats. Biochem. J., *37:* 508–513.

HART, E. B., V. E. NELSON AND W. PITZ 1918 Synthetic capacity of the mammary gland. I. Can this gland synthesize lysine? J. Biol. Chem., *36:* 291–307,

HEGSTED, D. M., R. C. MILLS, C. A. ELVEHJEM AND E. B. HART 1941 Choline in the nutrition of chicks. Ibid., *138:* 459–466.

KREHL, W. A., L. M. HENDERSON, J. DE LA HUERGA AND C. A. ELVEHJEM 1946a The relation of amino acid imbalance to niacin and tryptophan deficiency in growing rats. Ibid., *166:* 531–540.

KREHL, W. A., P. S. SARMA AND C. A. ELVEHJEM 1946b The effect of protein on the nicotinic acid and tryptophan requirement of the growing rat. Ibid., *162:* 403–411.

MELNICK, D., B. L. OSER AND S. WEISS 1946 Rate of enzymatic digestion of proteins as a factor in nutrition. Science, *103:* 326–329.

OSBORNE, T. B., AND L. B. MENDEL 1914 Amino acids in nutrition and growth. J. Biol. Chem., *17:* 325–349.

———— 1915 The amino acid minimum for maintenance and growth, as exemplified by further experiments with lysine and tryptophan. Ibid., *25:* 1–12.

ROSE, W. C. 1937 The nutritive significance of the amino acids and certain related compounds. Science, *86:* 298–300.

———— 1938 The nutritive significance of the amino acids. Physiol. Rev., *18:* 109–136.

WINTERNITZ, J. K. 1942 Some aspects of lysine deficiency in albino rats. Thesis submitted to the Department of Physiological Chemistry, Yale University, pp. 1–105.

1 Age 90 days, weight 350 gm, casein control diet (diet A) ad libitum.

2 Age 90 days, weight 144 gm, zein control diet with lysine (diet B) ad libitum.

3 Age 90 days, weight 61 gm, zein control diet with lysine (diet B) pair-fed with lysine-deficient animal no. 4.

4 Age 90 days, weight 36 gm, lysine-deficient diet (diet C) ad libitum.

THIAMINE AND RIBOFLAVIN INTAKES AND EXCRETIONS DURING PREGNANCY[1]

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THREE FIGURES

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It is generally considered that requirements for the various dietary essentials are higher during the latter half of pregnancy than during non-pregnant periods (Food and Nutrition Board of the National Research Council, '48). The present investigation was initiated in order to study the levels of intake of thiamine and riboflavin of primigravidae on self-selected diets, the corresponding rates of urinary excretion, and the test dose returns of these vitamins.

EXPERIMENTAL PROCEDURE

Fifteen women,[2] representing widely different racial, socio-economic and educational backgrounds, were studied during the latter half of pregnancy. Of these subjects, 13 were primigravidae, two being classed as gravida II because of previous abortions. The women received the usual dietary instructions from their physicians and clinic nutritionists. Other than this, no attempt was made to influence the selection or the quantity of food eaten or the amount of vitamin supplementation. All lived in their own homes and continued their normal activities.

The subjects were taught to weigh their own food, to keep accurate records of the amounts consumed, and to save repre-

[1] This study was supported in part by a grant from the National Dairy Council on behalf of the American Dairy Association.

[2] Twelve of the 15 women were patients at The Chicago Lying-in Hospital.

sentative samples of approximately one-fourth the amounts eaten. These were put in separate containers which were collected daily, as was the complete 24-hour urine output. In the case of 4 subjects, complete collections of feces, marked with carmine at the beginning and end of each period, were also made. Acetic acid and toluene were used as preservatives for the urine; acetic acid and ethyl alcohol for the feces.

Fourteen of the subjects were studied for 7-day periods during each of the last 5 months and one for a 7-day period during each of the last 4 months of pregnancy. At the end of each period composites were made of one-fifth of the amount of each food consumed and of the complete urinary excretions. Aliquots of each were analyzed for thiamine and riboflavin and, in the case of 4 subjects, for nitrogen. Weekly composites of fecal collections were analyzed for nitrogen.

Immediately after each 7-day period test doses of approximately 20 μg of riboflavin and 20 μg of thiamine [3] per kilogram of body weight were given orally on successive mornings with identical breakfasts.[4] On these days 4-hour urine specimens were collected. The percentage of the test dose returned was calculated after deducting the amount of the vitamin excreted during a 4-hour control collection made after a similar breakfast on the day preceding the administration of the first test dose.

The method of Hennessy and Cerecedo ('39) was used in the determination of thiamine in food composites; the method of Mickelsen, Condiff and Keys ('45) in urine samples. The microbiological method of Snell and Strong ('39), with certain modifications (Association of Vitamin Chemists, '47), was used for all riboflavin analyses. Total nitrogen was determined by the Kjeldahl method. Average daily intakes of nutrients

[3] The synthetic vitamins were generously supplied by Merck and Company.

[4] In cases when niacinamide test doses were given, the riboflavin test dose was given the first day, the niacinamide the second and the thiamine the third day immediately following the 7-day period.

TABLE 1

Weights and average intakes of essential nutrients of 18 subjects

TABLE 1

Weights and average intakes of essential nutrients of 15 subjects

SUBJECT	WT. RANGE [1]	CALORIES	PROTEIN	CALCIUM	IRON	VITAMIN A	ASCORBIC ACID [2]	THIAMINE	RIBOFLAVIN	NIACIN [3]
	kg		*gm*	*gm*	*mg*	*I.U.*	*mg*	*mg*	*mg*	*mg*
				(1.9–2.7) [4]	*(68–120)*			*(10.2–19.2)*	*(11.0–20.3)*	*(53.0–86.1)*
1	61–68	1700–2325	85–92	1.2–1.5	10–14	6,975–12,575	103–159	1.2–1.6	2.0–2.6	12.9–17.7
						(16,600–25,125)	*(180–290)*	*(4.5–4.9)*	*(5.8–6.9)*	*(36.8–43.1)*
2	57–58	1450–2025	89–124	1.6–2.3	13–21	11,600–20,125	105–215	1.2–1.6	2.5–3.5	11.8–18.1
						(9,650–11,675)	*(79–134)*	*(3.8–3.9)*	*(4.2–4.9)*	*(28.5–30.1)*
3	62–65	1350–1500	57–72	0.9–1.1	8–10	4,650–6,675	41–96	0.8–0.9	1.7–2.4	8.5–10.1
				(0.9–1.3)	*(10–29)*			*(0.7–4.5)*	*(1.6–5.2)*	*(14.1–23.8)*
4	59–64	1650–2100	62–74	0.8–1.2	10–11	5,300–10,200	27–59	0.7–1.5	1.6–2.6	11.2–14.5
							(354–818)	*(1.5–2.6)*	*(3.0–3.8)*	*(14.3–22.7)*
5	56–60	2150–2275	74–87	1.3–1.4	13–16	10,525–17,000	104–175	1.2–1.5	2.4–3.0	12.5–17.7
6	58–63	1750–1950	78–86	1.3–1.6	11–14	6,050–10,225	72–110	1.1–1.3	2.0–3.1	13.2–16.4
						(14,000–16,575)				
7	53–57	2025–2300	85–100	1.1–1.4	14–16	9,000–11,575	95–153	1.1–1.3	2.3–2.7	16.0–18.9
						(12,325–14,675)				
8	56–63	1600–2000	71–81	1.0–1.1	10–13	5,400–9,275	72–113	1.1–1.2	1.9–2.5	13.2–16.2
						(7,550–14,950)				
9	62–69	1375–2125	51–71	0.4–0.9	8–15	2,550–9,950	32–61	0.7–1.1	1.2–3.3	11.6–19.0
						(12,275–16,575)				
10	47–52	1350–1775	62–69	0.9–1.0	9–13	7,275–11,575	81–138	1.0–1.3	1.7–2.2	8.2–10.7
						(3,250–9,975)				
11	60–66	1400–1800	54–63	0.6–0.9	8–10	3,250–4,975	27–70	0.9–1.3	1.4–1.7	9.8–11.8
						(3,425–7,350)				
12	55–62	900–1175	30–47	0.2–0.3	5–8	1,175–3,425	6–19	0.5–0.6	0.5–0.8	7.2–11.3
				(0.3–0.4)	*(14–151)*	*(1,950–9,750)*				
13	54–61	700–1300	28–46	0.2–0.3	4–8	725–4,750	5–15	0.5–0.7	0.5–0.9	4.9–10.7
14	72–81	650–825	28–32	0.1–0.2	4–5	525–1,725	13–38	0.4–0.6	0.4–0.5	4.2–7.2
15	53–59	350–650	10–20	0.1–0.4	4–5	900–2,900	22–157	0.4–0.5	0.2–0.7	3.8–5.2
N.R.C.	allowance	2400	85	1.5	15	6,000	100	1.5	2.5	15

[1] Used to determine size of test doses. Weights were not taken under standard conditions but indicate gain during the period of study.

[2] Ascorbic acid from raw foods only.

[3] Niacin intakes for subjects 12, 13, 14, 15 are analyzed values (microbiological method).

[4] Italicized figures include supplementation and represent total intake.

other than thiamine and riboflavin were calculated for each period, using values published by the U. S. Department of Agriculture ('45) and by Taylor ('42).

RESULTS AND DISCUSSION

Average daily intakes of different nutrients

The weights of the subjects and the range of intakes of the different nutrients are presented in table 1. It will be noted that no diet met the allowances recommended by the Food and Nutrition Board of The National Research Council ('48) in all respects and during all periods.

Although the daily caloric intakes of subjects 1 and 5 approached the recommended 2,400 cal., the intakes of the remainder of the group were definitely lower, the average intake for the entire group being approximately 1,450 cal. per day. However, if the extremely low intakes of subjects 12, 13, 14 and 15 are disregarded, the average for subjects 1 through 11 is approximately 1,850 cal.

Subjects 12 through 15 were selected because interviews with the clinic nutritionist had indicated that their diets had been and would continue to be low in thiamine and riboflavin. These women neither drank milk nor ate eggs and meat in any but very small quantities. They also limited their carbohydrate intakes to avoid excessive weight gains. As a result, their caloric intakes averaged 1,000, 1,100, 750 and 500, respectively. Although weight gains on such intakes cannot be explained, other investigators (Winters and Leslie, '43; Ohlson et al., '48) studying the self-selected diets of women have encountered extremely low caloric intakes coupled with a failure to observe that the subjects were underweight.

The average daily protein intakes for the different subjects ranged from 10 to 124 gm, with those of only 5 subjects (1, 2, 5, 6, 7) meeting the suggested allowance. The nitrogen balances of 11 subjects are shown in table 2. Subject 15, with a daily protein intake which averaged 14 gm, was in negative nitrogen balance in every period, with an average daily nitrogen loss of 5.1 gm; subject 14, with a daily protein intake

TABLE 2

Average daily intakes, excretions and average test dose returns of thiamine and riboflavin; nitrogen balances

SUBJECT	THIAMINE			RIBOFLAVIN			NITROGEN			
	Ave daily intake [1]	Ave. daily excretion	Ave. test dose return	Ave. daily intake [1]	Ave. daily excretion	Ave. test dose return	Ave. daily intake [2]	Ave. daily excretion Urine [3]	Feces [4]	Balance
	mg	*mg*	%	*mg*	*mg*	%	*gm*	*gm*	*gm*	*gm*
1	16.50	5.00	14	17.50	12.60	69	14.0			
2	4.70	0.92	10	6.40	4.60	65	17.3			
3	3.90	0.63	12	4.50	2.20	44	10.2	8.9	1.0	+ 0.3
4	2.80	0.88	16	3.90	1.90	32	10.7	7.9	1.0	+ 1.8
5	2.10	0.43	10	3.50	2.30	44	13.3			
6	1.16	0.22	7	2.60	1.20	37	13.3	11.0	1.0	+ 1.3
7	1.21	0.24	5	2.51	1.03	31	15.0	11.4	1.0	+ 2.6
8	1.10	0.22	6	2.18	0.69	26	11.8	9.7	1.0	+ 1.1
9	0.85	0.19	5	2.08	0.82	16	9.4	9.5	1.0	— 1.1
10	1.16	0.23	8	1.87	0.62	34	10.5			
11	1.07	0.23	8	1.60	0.44	22	9.3	8.7	1.0	— 0.4
12	0.55	0.16	5	0.71	0.16	14	6.5	5.4	0.6	+ 0.5
13	0.66	0.16	5	0.69	0.07	9	5.8	4.8	0.6	+ 0.4
14	0.50	0.10	2	0.49	0.12	13	4.7	4.9	0.6	— 0.8
15	0.42	0.13	4	0.47	0.16	3	2.2	6.3	1.0	— 5.1

[1] Intakes of subjects 1 through 5 include supplementation with varying amounts of synthetic vitamins.

[2] Calculated values, with the exception of subjects 12 through 15.

[3] The authors were very kindly given access to the urinary nitrogen determinations on 7 subjects by the laboratories of Chicago Lying-in Hospital.

[4] A fecal nitrogen loss of 1 gm daily was assumed with the exception of subjects 12 through 15.

which averaged 29 gm, was in negative nitrogen balance in three out of 5 periods, which made her daily nitrogen loss average 0.8 gm. With one exception, the 5 subjects (3, 9, 11, 12, 13) whose daily protein intakes averaged from 36 to 65 gm had average nitrogen balances which ranged between — 0.5 and + 0.5 gm per day. The 4 subjects (4, 6, 7, 8) whose daily protein intakes averaged 67 gm or more stored over 1 gm of nitrogen per day, an amount which over a 5-month interval is more than sufficient to compensate for nitrogen loss on the day of delivery (Hunscher et al., '35). When these nitrogen balances were compared with the corresponding weight gains, discrepancies were observed in the case of certain subjects. Therefore, although these balances can be used as a general indication of the status of these individuals in regard to protein, they cannot be used to calculate protoplasmic mass.

The dietary calcium intakes of only two subjects (2, 6) met the recommended allowance; the average daily intakes, including supplementation, ranged from 0.1 to 2.7 gm. The dietary iron intakes of only three subjects (2, 5, 7) met the recommended allowance; the average daily intakes, including supplementation, ranged from 4 to 120 mg.

The diets of subjects 1 through 10 met the recommended allowance for vitamin A and in addition 6 of these subjects received supplements of this vitamin. The diets of subjects 11 through 15 did not meet the recommended allowance for vitamin A but three of these subjects received sufficient supplementation to bring their average daily intakes to 6,000 I.U. Therefore, only two subjects had vitamin A intakes which failed to meet the recommended allowance.

Only three subjects did not supplement their diets with at least 300 I.U. of vitamin D. One of the three (6) consistently drank enough irradiated milk to supply 350 I.U. per day; the other two (14, 15) used practically no milk and therefore had no appreciable food source of vitamin D.

The dietary ascorbic acid intakes of 5 subjects (1, 2, 5, 7, 10) met the recommended allowance. The average daily intakes, including supplementation, ranged from 5 to 818 mg.

None of the subjects had a dietary thiamine intake which met the recommended allowance during all 5 periods. However, when supplementation is included, subjects 1 through 5 were receiving more than 1.5 mg per average day. The average intakes, including supplementation, ranged from 0.4 to 19.2 mg per day. On the other hand, the dietary riboflavin intakes of 4 subjects (2, 5, 6, 7), all of whom drank at least three glasses of milk per day, met the recommended allowance. When supplementation is included, three additional subjects (1, 3, 4) also had intakes of 2.5 mg or more. The average daily riboflavin intakes, including supplementation, ranged from 0.2 to 20.3 mg. In the case of niacin, the dietary intake of 7 subjects (1, 2, 5, 6, 7, 8, 9) and, when supplementation is included, of two additional subjects (3, 4), met the recommended allowance. The average daily niacin intakes, including supplementation, ranged from 4 to 86 mg.

In general, the diets of all subjects were definitely lower than the suggested allowances for calories and the diets of only 5 of the 15 (1, 2, 5, 6, 7) approached the suggested allowances for essential nutrients.

Course of pregnancies and condition of infants

The course of pregnancy in all cases was uneventful, and the deliveries were normal. The 12 infants delivered at the Chicago Lying-in Hospital were examined by the pediatrician and all found to be free from defects. Although records of the three other infants were not available to us, there was no indication of any abnormalities whatsoever. Furthermore, no deviations from the normal were noted in the x-rays of the 4 infants whose mothers had the poorest diets.

The birth lengths and weights of the infants showed no correlation with the quality of the diets of the mothers, notwithstanding the fact that these diets differed greatly in nutritive value. When the diets were evaluated by the method of Burke and Stuart ('38), those of 8 subjects (1 through 8) were rated

as excellent or good; those of the other 7 as fair, poor or very poor. The average birth lengths of the infants of these two groups were 50.4 and 50.6 cm respectively; the corresponding average birth weights, 3,145 and 3,499 gm. When the diets were evaluated on the basis of protein content only, using the standards of Burke et al. ('43c), those of 6 subjects (1, 2, 5, 6, 7, 8) were rated as excellent or good; those of the other 9 as fair to very poor. The average birth lengths of the infants of these two groups were 50.2 and 50.7 cm, respectively; the corresponding average birth weights, 3,142 and 3,422 gm.

A follow-up study made on 14 of the infants when they were from 6 months to three years of age showed that all were in good health and had had no serious illnesses of any kind, with the possible exception of one who was under observation for a suspected allergic condition. The group as a whole had made 132% of its expected gain in weight and 113% of its expected gain in height when compared with Woodbury's standards ('21). No relationship could be found between the growth of the infants and the quality of the mothers' diets during pregnancy.

It was not the objective of this study to determine the effect of diet on the course of pregnancy and the condition of the infant. However, it is worth noting that the results are similar to those obtained by Sontag and Wines ('47), Brzezinski et al. ('47), Northrop and Piper ('47) and Dieckmann et al. ('48), and are not in accord with those of Ebbs et al. ('41) and Burke et al. ('43a, b, c).

Because of the limited number of subjects studied, these results do not justify the conclusion that the diet of the mother during pregnancy has no effect on the development of the infant or the health of the mother. It should also be kept in mind that since the subjects of the present study were primigravidae, these results do not measure the cumulative effects of such diets in the case of more than one pregnancy. However, if the prenatal diet did have an effect in the case of these subjects, more rigorous criteria would have been required for its demonstration.

Thiamine and riboflavin utilization

The average daily thiamine and riboflavin intakes and excretions are presented in table 2. The excretions of both vitamins increased directly with increased intakes, as is shown by a correlation coefficient of 0.98 for thiamine and 0.99 for riboflavin. There was no consistent decrease in the daily thiamine excretions with advancing pregnancy, as was reported by Siddall and Mull ('45).

Both urinary thiamine and riboflavin excretions of the 5 subjects who supplemented their diets with synthetic vitamins indicated that their intakes obviously exceeded their needs.

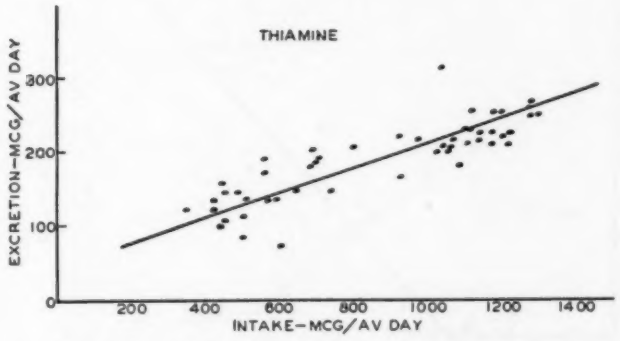

Fig. 1 Average daily intakes and excretions of thiamine.

Therefore, only the data from the 10 subjects who used no supplementary vitamins are included in the discussion which follows.

When thiamine excretions were plotted against thiamine intakes, a linear relationship was obvious (fig. 1). If the "point of minimum excretion" designated by Holt et al. ('49) was attained, the data below that point were too limited to permit its demonstration. This is not surprising, since these authors state that the point of minimum excretion in adult subjects previously studied in their laboratory was reached when the thiamine intake was 0.6 mg per day; in the present

study relatively few of the subjects had intakes below this amount.

A comparison of the urinary thiamine excretions of these pregnant women with those found by Hathaway and Strom ('46) in non-pregnant women of comparable ages on natural diets and by Oldham et al. ('46) and Daum et al. ('48) shows that on all intakes of less than 1 mg per day the pregnant

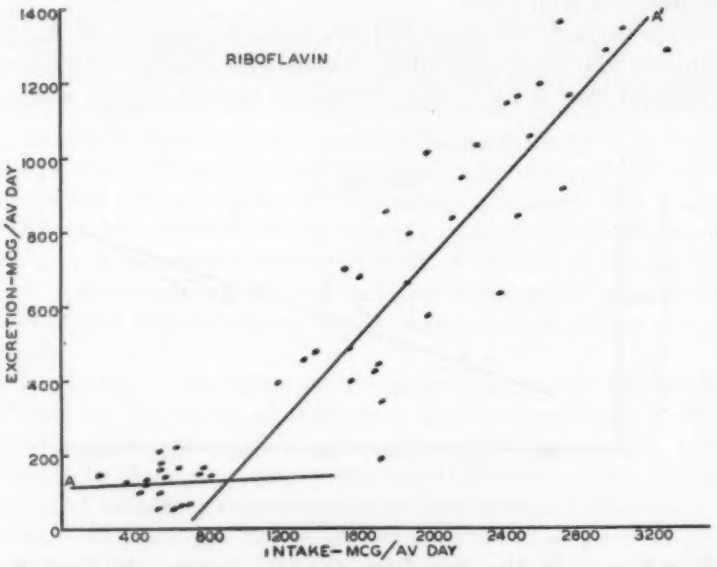

Fig. 2 Average daily riboflavin intakes and excretions of primigravidae.

women were excreting from two to three times as much thiamine as the non-pregnant women on similar intakes. A possible explanation for this is the fact that the thiamine intakes, when expressed as milligrams per 1,000 cal., are at least as high and for the most part relatively much higher in the case of the pregnant women with low thiamine intakes than in the non-pregnant women with similar thiamine intakes.

The percentage returns of oral test doses of approximately 1 mg of thiamine are shown in table 2. The returns of subjects

who supplemented their diets were higher in every case than those of subjects who did not. In general, the responses were of the same magnitude as those found by Oldham et al. ('46) in non-pregnant women on similar intakes. A definite decrease in the percentage of the test dose returned with advancing pregnancy, which might have been expected according to the

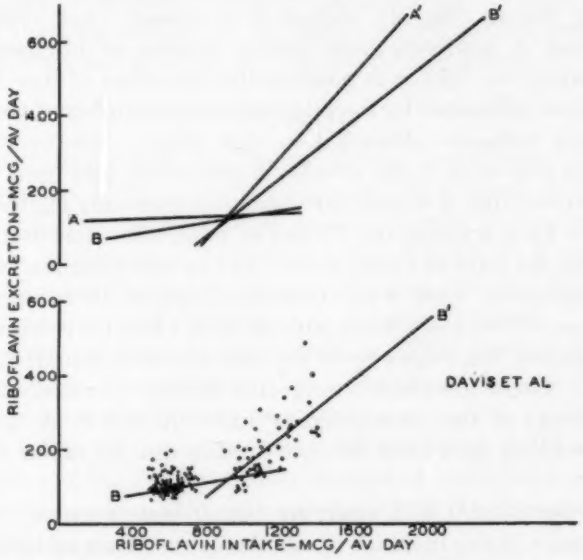

Fig. 3 A comparison of regression lines representing data from primigravidae and from non-pregnant women; average daily riboflavin intakes and excretions of non-pregnant women.

reports of Lockhart et al. ('43) and Darby et al. ('48), was noted in only 4 subjects (6, 8, 14, 15).

Since the test dose returns were similar to those of non-pregnant women, with no consistent decrease as pregnancy advanced, and since the average daily excretions on intakes below 1 mg were significantly higher than those of non-pregnant women, these data offer no evidence that there is an increased need for thiamine during a normal pregnancy.

When riboflavin excretions were plotted against riboflavin intakes, there appeared to be two population groups, one consisting of the 4 subjects (12, 13, 14, 15) with the poorest diets, the other of the remainder of the non-supplemented group (6, 7, 8, 9, 10, 11). Regression lines drawn for these two groups according to the equation $y = a + bx$ had significantly different slopes ($t = 5.62$) and intersected at an intake of approximately 900 μg (fig. 2). Since it is known that riboflavin excretion is relatively high during periods of nitrogen loss (Oldham et al., '47), it is possible that the slope of line A may have been influenced by the relatively large number of negative nitrogen balances obtaining in this group. However, it is obvious that even if the riboflavin excretions had been lower, the slope of line A would have been changed only slightly and that we have reached the "point of minimum excretion."

When the data of Davis et al. ('46) on non-pregnant women of comparable ages were treated similarly there was also evidence of two population groups, and when regression lines were drawn the slopes were significantly different ($t = 3.41$; fig. 3). These lines also intersected at approximately 900 μg. The slopes of the corresponding lines (A and B, A′ and B′) representing data from the two studies did not differ significantly.

Brewer et al. ('46), studying young non-pregnant women, reported a sharp increase in riboflavin excretion on intakes of over 2 mg, as contrasted with a gradual increase on lower intakes. Regression lines for the data from these two groups, however, intersected at approximately 1.4 mg. The discrepancy between these data and those of Davis et al. ('46) appears to be due to the fact that Davis' subjects showed a sharp increase in excretions on intakes of 1.2 mg, whereas Brewer's subjects did not show such an increase until the intakes were higher. Unfortunately, practically none of the subjects of the present study had intakes of this particular magnitude.

The percentage return of riboflavin test doses increased with increasing intakes. As was the case with thiamine, definite

decreases with advancing pregnancy were not found consistently.

Since the riboflavin test dose returns showed no indication of an increased need for riboflavin with advancing pregnancy, and since the point of minimum excretion of riboflavin in the case of pregnant women was attained on intakes which were no higher than those found for non-pregnant women, it appears that the utilization of riboflavin in normal pregnant women does not differ from that in the non-pregnant.

SUMMARY

The thiamine and riboflavin intakes, excretions and test dose returns of 15 pregnant women on self-selected diets were determined. Intakes of other nutrients were calculated.

With the exception of calories, the intakes of 5 subjects approximated the recommended allowances of the Food and Nutrition Board of the National Research Council. The intakes of all nutrients by the remainder of the group were definitely below the recommended allowances, with 4 of the subjects having extremely poor diets.

The course of the pregnancies, the condition of the infants at birth, and the postnatal development of the infants did not appear to be correlated with the quality of the prenatal diets of the mothers.

When compared with data on non-pregnant women on similar intakes, neither the returns of thiamine and riboflavin test doses nor the daily excretions of these vitamins indicated an increased need for them during a normal pregnancy.

ACKNOWLEDGMENTS

We wish to express our appreciation for the cooperation of Dr. W. J. Dieckmann and Miss Ella Jane Meiller of the Chicago Lying-in Hospital. The technical assistance of Elsie O'R. Sears and Mildred M. Flaska is also gratefully acknowledged.

LITERATURE CITED

ASSOCIATION OF VITAMIN CHEMISTS 1947 Methods of Vitamin Assay. Interscience Publishers, Inc., New York.

BREWER, W., T. PORTER, R. INGALLS AND M. A. OHLSON 1946 The urinary excretion of riboflavin by college women. J. Nutrition, *32:* 583.

BRZEZINSKI, A., Y. M. BROMBERG AND K. BRAUN 1947 Riboflavin deficiency in pregnancy. Its relationship to the course of pregnancy and to the condition of the foetus. J. Obst. Gyn. Brit. Empire, *54:* 182.

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———— 1943b The influence of nutrition during pregnancy upon the condition of the infant at birth. J. Nutrition, *26:* 569.

BURKE, B. S., V. V. HARDING AND H. C. STUART 1943c Nutrition studies during pregnancy. IV. Relation of protein content of mother's diet during pregnancy to birth length, birth weight and condition of infant at birth. J. Pediat., *23:* 506.

BURKE, B. S., AND H. C. STUART 1938 A method of diet analysis. Ibid., *12:* 493.

DARBY, W. J., R. O. CANNON AND M. N. KASER 1948 The biochemical assessment of nutritional status during pregnancy. Obst. Gyn. Survey, *3:* 704.

DAUM, K., W. W. TUTTLE, M. WILSON AND H. RHOADS 1948 Influence of various levels of thiamine intake on physiologic response. II. Urinary excretion of thiamine. J. Am. Diet. Assn., *24:* 1049.

DAVIS, M. V., H. G. OLDHAM AND L. J. ROBERTS 1946 Riboflavin excretions of young women on diets containing varying levels of the B vitamins. J. Nutrition, *32:* 143.

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EBBS, J. H., F. F. TISDALL AND W. A. SCOTT 1941 The influence of prenatal diet on the mother and child. J. Nutrition, *22:* 515.

FOOD AND NUTRITION BOARD, NATIONAL RESEARCH COUNCIL 1948 Recommended Dietary Allowances, Rev. Circular Series 129.

HATHAWAY, M. L., AND J. E. STROM 1946 A comparison of thiamine synthesis and excretion in human subjects on synthetic and natural diets. J. Nutrition, *32:* 1.

HENNESSY, D. J., AND L. R. CERECEDO 1939 The determination of free and phosphorylated thiamine by a modified thiochrome assay. J. Am. Chem. Soc., *61:* 179.

HOLT, L. E., JR., R. L. NEMIR, S. E. SNYDERMAN, A. A. ALBANESE, K. C. KETRON, L. P. GUY AND R. CARRETERO 1949 The thiamine requirement of the normal infant. J. Nutrition, *37:* 53.

HUNSCHER, H. A., F. C. HUMMELL, B. N. ERICKSON AND I. G. MACY 1935 Metabolism of women during the reproductive cycle. VI. A case study of the continuous nitrogen utilization of a multipara during pregnancy. Ibid., *10:* 579.

LOCKHART, H. S., S. KIRKWOOD AND R. S. HARRIS 1943 The effect of pregnancy and puerperium on the thiamine status of women. Am. J. Obst., *46:* 358.

MICKELSEN, O., H. CONDIFF AND A. KEYS 1945 The determination of thiamine in urine by means of the thiochrome technique. J. Biol. Chem., *160:* 361.

NORTHROP, M. W., AND G. M. PIPER 1947 A study of diets of patients in a prenatal clinic with an attempt to correlate dietary adequacy with physical findings. Northwest Med., *46:* 294.

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OLDHAM, H., E. LOUNDS AND T. PORTER 1947 Riboflavin excretions and test dose returns of young women during periods of positive and negative nitrogen balance. Ibid., *34:* 69.

SIDDALL, A. C., AND J. W. MULL 1945 Thiamine status during pregnancy. Am. J. Obst., *49:* 672.

SNELL, E. E., AND F. M. STRONG 1939 A microbiological assay for riboflavin. Ind. Eng. Chem., Anal. Ed., *11:* 346.

SONTAG, L. W., AND J. WINES 1947 Relation of mothers' diets to status of their infants at birth and in infancy. Am. J. Obst., *54:* 994.

TAYLOR, C. M. 1942 Food Values in Shares and Weights. The Macmillan Company, New York.

UNITED STATES DEPARTMENT OF AGRICULTURE 1945 Tables of Food Composition. Misc. Publ. no. 572.

WINTERS, J. C., AND R. E. LESLIE 1943 A study of the diet and nutritional status of women in a low-income population group. J. Nutrition, *26:* 443.

WOODBURY, R. M. 1921 Statures and Weights of Children Under Six Years of Age. Children's Bureau, U. S. Dept. of Labor, Bull. 87.

CORRELATION OF URINARY EXCRETION OF RIBOFLAVIN WITH DIETARY INTAKE AND SYMPTOMS OF ARIBOFLAVINOSIS [1]

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The urinary output of riboflavin by individuals on diets containing varying surpluses of riboflavin varied greatly, but as the intake of riboflavin was reduced these variations became smaller and at very low levels of intake the amounts

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LITERATURE CITED

ASSOCIATION OF VITAMIN CHEMISTS 1947 Methods of Vitamin Assay. Interscience Publishers, Inc., New York.

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excreted became quite uniform. Thus the per cent of ingested riboflavin excreted can be correlated with the adequacy of the amount of riboflavin in the diet.

EXPERIMENTAL

The 5 basal diets used during the periods when urine was collected for these studies provided amounts of riboflavin ranging from 0.55 to 1.6 mg per day. These diets when supplemented served as examples of higher levels of riboflavin intake. Table 1 summarizes the thiamine and riboflavin content of the diets to be discussed. Group A refers to subjects on a depleted diet, group B to subjects who were supplemented at the beginning of an experiment, and group C to subjects who ate the regular hospital diet.

Our nutritional research of the past 6 years can be divided into two main phases: the first was concerned with a study of human thiamine and riboflavin requirements, with emphasis on the thiamine requirement; the second was devoted exclusively to a study of riboflavin.

In the first project (Horwitt, Liebert, Kreisler and Wittman, '48) 36 male patients were divided into three groups designated as groups A, B and C, respectively, on June 6, 1943. Each person in the A group received a daily diet containing approximately 2,200 calories. This diet contained about 0.4 mg of thiamine and *0.85 mg* of riboflavin. The subjects in the B group received the same diet except that a daily supplement of a special yeast extract was given which contained approximately 6 mg of thiamine and 1.3 mg of riboflavin. The members of the C group received the regular hospital diet, which contained about 1 mg of thiamine and *1.6 mg* of riboflavin. At the end of the second year (June 5, 1945), the basal diet of the B group was changed by further restricting the meat and flour allotment. The yeast supplement was withdrawn from this group at that time, so that the diet then provided only 0.2 mg of thiamine and *0.75 mg* of riboflavin.

Supplementation with riboflavin of most of the subjects who had received 0.75 and 0.85 mg of riboflavin per day was

TABLE 1

Basic plans of diets used in studies of thiamine and riboflavin

GROUP	VITAMIN SUPPLEMENT	PROJECT I			GROUP	VITAMIN SUPPLEMENT	PROJECT II	
		Periods diets were fed					Periods diets were fed	
		6/43 to 6/45	6/45 to 9/46 [1]	11/45 to 10/46			2/47 to 5/47	5/47 to 10/48 [2]
		mg/day	*mg/day*	*mg/day*			*mg/day*	*mg/day*
A	Thiamine	0.40	0.40	6.40	A	Riboflavin	1.10	0.55
	Riboflavin	0.85	0.85	2.15				
B	Thiamine	6.40	0.20	6.20	B	Riboflavin	1.10	2.55 [3]
	Riboflavin	2.15	0.75	2.05				
C	Thiamine	1.00	1.00	1.00	C	Riboflavin	1.60	1.60
	Riboflavin	1.60	1.60	1.60				

[1] Most of the subjects in group B were supplemented after only 6 months on the depleted diet because of the appearance of signs of thiamine deficiency. Group A subjects were not supplemented until after they had been on their diet for from 27 to 39 months.

[2] The range of depletion periods for subjects in group A varied from 9 to 17 months. The riboflavin supplement provided at the end of these periods was either 2, 4 or 6 mg per day.

[3] Increased to 3.55 mg per day on July 20th, 1948, due to increase in riboflavin supplement from 2 to 3 mg per day.

accomplished by giving the yeast extract referred to above, thus providing 1.3 mg of riboflavin. This raised the daily riboflavin intake to *2.05* and *2.15 mg* per day, respectively. The dates of supplementation extended from November 1945 to September 1946.

The diets used in the second project were planned to be complete in all members of the vitamin B complex except riboflavin. The choice of subjects and the extent of the dietary control are described by Horwitt, Sampson, Hills and Steinberg ('49) and Horwitt, Hills, Harvey, Liebert and Steinberg ('49). A diet providing *1.1 mg* of riboflavin per day was fed for a three-month period (February to May 1947) to 30 male subjects. Fifteen of these subjects were then placed on a diet which provided 2,200 calories, 57 gm of protein, 65 gm of fat and *0.55 mg* of riboflavin. This schedule was adhered to for periods ranging from 9 to 17 months. At the same time, 14 of the original subjects were fed the same diet plus a supplement of 2 mg of riboflavin per day (total *2.55 mg*). This supplementation was continued for 15 months, at which time (July 1948) the supplement was increased to 3 mg of riboflavin (total *3.55 mg* per day).

In an effort to learn something about the rate at which tissues depleted of riboflavin can be saturated, different amounts of riboflavin were given to subjects on the 0.55-mg diet at the termination of their depletion periods. Accordingly, 4 subjects received 6 mg once a day, three subjects received 2 mg twice a day, 4 subjects received 2 mg once a day, and three subjects received 2 mg three times a day.

All urine samples were collected in brown glass bottles which contained 5 ml of glacial acetic acid and a few milliliters of toluene. The urine was protected from light during handling and stored in a refrigerator until analyzed. In recent years we have used the methods of determining riboflavin in urine developed by Roberts and Snell ('46) for the microbiological and Slater and Morell ('46) for the fluorometric determinations. Prior to 1946 most urine analyses were made according to the microbiological method of Snell and Strong ('39). The

effects of urea on the microbiological analysis were controlled in the present work (Isbell, Wooley and Fraser, '41).

The clinical evaluations of the symptoms of ariboflavinosis observed have been summarized elsewhere (Horwitt, Hills, Harvey, Liebert and Steinberg, '49).

RESULTS

Since study of the diet which provided 0.55 mg of riboflavin per day produced incontrovertible signs of riboflavin deficiency, the urinary data obtained during this study will be discussed in some detail. Tables 2 and 3 give the individual results of the 24-hour and 4-hour excretions, respectively, of the subjects who were on this diet. The data obtained during the preliminary period, after the subjects had been on the 1.1-mg diet for three months, are included in each table. The volumes of urine obtained in each sample are also listed in these tables for comparison with the data reported by Feder, Lewis and Alden ('44), who claimed that amounts of riboflavin per milliliter of urine show less variation than the total amounts excreted.

24-hour urinary excretions on 0.55-mg diet and after supplementation with 6 mg of riboflavin per day

At the time the subjects were being selected (February 1947) the average hospital patient was excreting approximately 400 μg of riboflavin per day. At the end of the preliminary period (February to May 1947), during which the subjects had been on a diet which provided 1.1 mg of riboflavin per day, those subjects selected to be members of the A group excreted an average of 112 ± 75 μg of riboflavin per day (table 2). Seven days after the experimental diet (0.55 mg per day) was started, the A group average dropped to a figure of 49 ± 19 μg per day, which persisted for many months. This would indicate that no riboflavin reserve could have been retained or accumulated during the three months on the diet of 1.1 mg of riboflavin per day. After 9 months on the A group diet, an

TABLE 2

Urinary excretion of riboflavin in 24 hours. The diets indicated were used successively

Duration of diet in weeks at time of analysis	13		1		7		15		37		43		57		64	
Riboflavin intake, mg/day	1.1		0.55		0.55		0.55		0.55		0.55		0.55		0.55	
Date	4/21/47		5/14/47		6/26/47		8/19/47		1/14/48		3/5/48		6/11/48		7/30/48	
SUBJECT	*ml*	*μg*	*ml*	*μg*	*ml*	*μg*	*ml*	*μg*	*ml*	*μg*	*ml*	*μg*	*ml*	*μg*	*ml*	*μg*
A1	1,950	330	1,840	40	1,180	36	780	40	1,620	35						
2	2,540	75	1,380	47	1,010	38	580	50	2,255	49	2,250	38	2,890	37	1,520	35
3	1,520	71	890	25	920	28	700	54	1,320	30	1,300	28				
4	1,180	145	3,780	94	3,570	40	1,990	55	2,100	40	1,360	38	3,040	27	1,340	42
5	2,410	250	1,770	56	970	74	670	50	1,250	33	250	17				
6	1,420	40	1,000	28	1,230	23	1,520	44	2,295	34	1,410	18	2,170	32	2,230	21
7	970	77	1,980	42	2,960	52	860	30	1,880	27	2,045	49	2,550	56	1,660	14
8	2,060	112	620	30	1,000	28	750	54	1,520	38						
9	790	89	2,550	72	1,330	73	650	59	2,150	44	1,910	48	1,740	47	1,800	30
10	1,460	99	1,140	46	780	33	1,120	44	2,790	35	2,550	38	3,070	36	1,740	23
11	2,210	65	1,700	28	1,640	58	1,320	78	2,680	38	2,550	38	2,770	22	2,730	34
12	1,385	80	1,520	49	1,140	56	555	50	1,580	36	1,030	31	1,940	21	1,580	26
13	1,250	96	1,200	43	560	55	590	46	1,320	36	1,610	45	2,120	21	1,280	25
14	1,160	104	2,180	63	1,310	68	640	67	1,125	43	1,120	53	2,230	26	2,250	23
15	1,500	54	3,170	76	1,590	85	615	42	2,820	30	1,480	54	1,700	20	1,800	26
Average		112		49		50		51		37		38		31		27
Standard deviation		± 75		± 19		± 19		± 11		± 6		± 12		± 11		± 7

Duration of diet in weeks at time of analysis	13		1		7		15		37		43		1.5[1]	
Riboflavin intake, mg/day	1.1		2.55		2.55		2.55		2.55		2.55		3.55	
Date	4/21/47		5/14/47		6/26/47		8/19/47		1/14/48		3/5/48		7/30/48	
SUBJECT	*ml*	*μg*	*ml*	*μg*	*ml*	*μg*	*ml*	*μg*	*ml*	*μg*	*ml*	*μg*	*ml*	*μg*
B1	1,540	42	1,930	835	660	1,075	715	940	1,750	978	1,580	894	1,200	1,670
2	670	31	900	595	1,170	845	370	578						
3	1,640	160	1,220	945	1,510	1,115	705	618	1,380	1,033	1,740	745	1,920	1,875
4	990	42	1,110	640	710	945	910	775	1,640	801	2,130	702	1,850	1,960
5	1,820	78	1,640	650	4,690	1,180	2,340	1,080	2,930	315	2,100	1,200	2,370	1,575
6	2,330	97												
7	755	52	3,010	601	1,400	618	1,010	825	1,600	562	1,400	688	1,860	1,825
8	1,220	106	1,520	655	1,410	1,110	760	1,100	1,780	958	1,400	745	1,520	1,718
9	1,585	100	1,795	686	860	865	765	934	1,820	1,048	1,440	1,295	1,400	1,890
10	1,540	135	3,030	1,388	1,450	1,440	750	890	2,460	969				
11	1,620	99	2,010	620	1,890	835	1,520	1,020	2,500	890	2,106	1,165	2,280	1,825
12	950	47	660	207	1,770	572			2,445	680	500	301	2,280	880
13	1,930	71	2,410	785	2,610	154	1,140	920	1,860	955	1,780	1,085	1,870	1,935
14	2,160	66	2,700	912	4,410	1,085	2,450	1,100	2,390	970	2,100	783		
Average		80		732		911		898		847		873		1,715
Standard deviation		± 36		± 258		± 314		± 167		± 212		± 278		± 301

[1] Supplement increased after 62 weeks on 2.55-mg intake of riboflavin.

TABLE 3

Urinary excretion of riboflavin during the 4 hours after the subcutaneous administration of 1 mg of riboflavin

Duration of diet in weeks at time of analysis	13		8		37		70		74–80	
Riboflavin intake, mg/day	1.1		0.55		0.55		0.55		0.55	
Date	4/23/47		6/29/47		1/19/48		8/13/48		9/2/48 to 10/26/48	
SUBJECT	*ml*	*μg*	*ml*	*μg*	*ml*	*μg*	*ml*	*μg*	*ml*	*μg*
A1	640	48	195	18	575	14				
2	450	49	335	14	630	14	740	13		
3	280	48	150	10	465	11				
4	575	92	220	20	480	22	300	16	653	20
5	710	84	95	8	290	6				
6	315	14	80	12	655	23	780	12	540	5
7	255	130	365	20	550	16	700	17	800	33
8	300	31	370	13	210	17				
9	700	99	110	26	620	30	380	29	565	6
10	450	53	300	14	665	16	100	5	345	6
11	735	103	545	49	790	16	680	24	730	6
12	210	69	125	14	590	14	620	9	200	6
13	510	94	88	56	180	28	480	13	520	20
14	400	165	460	51	1,010	25	280	30	670	40
15	605	131	120	20	600	26	650	10	880	2
Average		81		23		18		16		14
Standard deviation		± 40		± 15		± 7		± 8		± 13

Duration of diet in weeks at time of analysis	13		8		37		70	
Riboflavin intake, mg/day	1.1		2.55		2.55		3.55 [1]	
Date	4/23/47		6/29/47		1/19/48		8/13/48	
SUBJECT	*ml*	*μg*	*ml*	*μg*	*ml*	*μg*	*ml*	*μg*
B1	690	102	250	268	660	242	300	271
2	522	29	110	226				
3	220	87	120	305	490	300	680	392
4			85	450	600	298	440	562
5	450	82	205	325	705	204	600	454
6	500	50						
7	110	43	170	195	215	255	870	324
8	210	42	195	224	925	164	860	382
9	590	68	450	112	780	419	500	302
10	715	43	410	1,056	900	408		
11	250	73	260	194	525	240	850	420
12	260	28	300	178	490	284	500	238
13	315	46	360	182	380	202	790	387
14	624	49	310	432	215	289		
Average		57		319		275		373
Standard deviation		± 22		± 233		± 74		± 90

[1] Supplement increased after 62 weeks on 2.55-mg intake of riboflavin.

average excretion of 37 ± 6 μg per day was recorded and no further significant change was obtained during the following 8 months of the depletion period.

The individual results obtained during the last weeks on the preliminary diet of 1.1 mg per day may be significant. Before the feeding of the 0.55-mg diet was started, the 15 subjects in the A group excreted, as was stated, an average of 112 ± 75 μg per day. Only 4 of these subjects had excretions which were equal to or higher than the average. At that time these subjects, A1, A4, A5 and A8, excreted daily 330, 145, 250 and 112 μg of riboflavin, respectively. The excretions of these subjects in later months were not significantly different from those of the other subjects in the A group, but the first three subjects to show any clinical signs of riboflavin deficiency were A1, A5 and A8. In other words, the three subjects who showed the earliest and most obvious clinical signs (traumatic changes in the skin around the oral angles and severe inflammation of the scrotum) had excreted a larger proportion of the riboflavin ingested during the period on the 1.1-mg diet than the average of their group. Whether this is a coincidence or a significant observation remains to be determined. Also of possible significance is the fact that all three of these subjects had auburn beards, which raises the question of individual variability due to differences in texture of the skin.

When it became advisable to expedite the recovery of the three subjects on the 0.55-mg diet who had developed the earliest skin lesions, they were supplemented with three 2-mg tablets of riboflavin once a day. On this therapy, which clinically was spectacularly efficacious, the daily urinary excretions increased very rapidly. Thus, for example, subject A5, who was supplemented on March 1st, starting at an excretion level of 33 μg per day, increased his excretion after one day on the 6-mg supplement to 1,100 μg and after 8 days to 2,590 μg of riboflavin. Interim samples were not obtained on subject A1, but after 10 weeks of supplementation he excreted 3,200 μg in 24 hours. Subject A8 excreted 2,030 μg after only 4 days of supplementation. That the ingestion of large amounts of water

will increase the excretion of riboflavin when it is supplied in excess of requirements was frequently observed. For example, subject A8 on the 17th day of supplementation excreted 3,900 μg with a urine volume of 3,420 ml, and 6 days later, when the 24-hour urine volume had dropped to 850 ml, only 1,870 μg. A similar correlation between the amounts of urine and riboflavin excreted was not apparent when the diet was low in riboflavin (see table 2).

Urinary excretion during 4 hours following subcutaneous administration of 1 mg of riboflavin to subjects when on a 0.55-mg diet and after supplementation with varying amounts of riboflavin

After the three-month preliminary period on the diet containing 1.1 mg of riboflavin per day, the 4-hour excretion test gave results (table 3) that averaged 81 ± 40 μg of riboflavin for the subjects who were to go on the 0.55-mg diet and 57 ± 22 μg for the subjects who were to receive the 2-mg supplement. These averages may be compared with the average of 208 ± 72 μg for the group which had been receiving a diet containing approximately 1.6 mg of riboflavin per day. This indicates that a marked difference in the urinary excretion of ingested riboflavin occurs somewhere between intakes of 1.1 and 1.6 mg of riboflavin per day. The desire to keep the intake of riboflavin constant during the depletion periods resulted in relatively few 4-hour tests being scheduled until after the final supplementations were started. Therefore, this test was not performed again until after the diet containing 0.55 mg per day had been fed for 8 weeks. At that time, the 4-hour excretion of the subjects on the 0.55-mg diet had dropped to an average of 23 ± 15 μg, while the average of the control group who were receiving the same diet plus a 2-mg supplement had risen to 319 ± 233 μg.

To determine whether or not the tissues can absorb riboflavin more efficiently when smaller or divided doses are given, 10 subjects from the group which had been on the 0.55-mg diet

for 16 months were supplemented as follows: 4 subjects were given 2 mg once a day, three subjects 2 mg twice a day, and three subjects 2 mg three times a day. All of these subjects had dropped to approximately the same 4-hour excretion level of 16 ± 8 μg during the 16th month of the depletion diet. (The analytical accuracy of data on urine samples showing less than 15 μg of riboflavin is questionable.) Relatively little difference was obtained among the 4-hour excretions at these three levels up to the 4th day, when the averages were 53 ± 34, 117 ± 86 and 53 ± 11 μg for the groups receiving 2 mg, 4 mg and 6 mg, respectively. In later analyses the amounts of the ingested riboflavin excreted were proportional to the size of the daily dose administered. Those who were supplemented with only 2 mg of riboflavin took 38 days to attain a peak excretion of 320 ± 28 μg. (The control group, which had been supplemented with 2 mg of riboflavin, had attained a similar average of 319 μg. See table 2.) Those depleted subjects who received 2 mg twice a day reached a maximum of 399 ± 144 μg in 25 days, and those receiving 2 mg three times a day reached a maximum 4-hour excretion of 565 ± 125 μg within 15 days. Unfortunately, only one subject (A2) was continued on this test after obtaining 6 mg of riboflavin in one dose (20 hours before the test of urine excretion). This subject reached an excretion level of 240 μg in 4 days. This fact, combined with the observation that the three other daily doses used were not more effective in increasing the riboflavin excretion during the first 4 days, leaves the impression that a single dose of 6 mg of riboflavin is as effective for therapeutic purposes as three 2-mg doses, given at 4-hour intervals, during the important early periods of supplementation.

Urinary excretion of riboflavin at other levels of riboflavin intake

The average 24-hour urinary excretion of 42 subjects who were eating the hospital diet which contained approximately 1.6 mg of riboflavin was 434 ± 185 μg. The excretion obtained

after the 4-hour test on 42 subjects receiving this diet was 227 ± 140 μg.

The average 24-hour excretion of 12 subjects who were changed from a diet providing 1.6 mg riboflavin to one containing 0.85 mg (Horwitt et al., '48) dropped from 399 ± 138 μg to 133 ± 52 μg in 10 weeks. Four weeks later the average daily excretion attained the plateau level of 76 ± 38 μg, which persisted with only minor variations for the next two years. The 4-hour test of the subjects on this diet provided data which indicated that the subjects had dropped to a plateau level in 11 weeks. The excretion at that time in the 4-hour test was 81 ± 41 μg.

The 11 subjects who received the 0.75-mg diet (Horwitt et al., '48) had previously been receiving a supplement for two years, during which period their intake had been 2.15 mg of riboflavin per day. On the latter level their 24-hour and 4-hour plateaus of excretion had attained averages of 714 ± 293 and 297 ± 124 μg, respectively. Three weeks after the commencement of the 0.75-mg diet, these average levels had dropped to 180 ± 38 and 60 ± 34 μg for the 24-hour and 4-hour tests, respectively. The 24-hour results had reached the minimum level of 70 ± 15 μg 9 weeks later, which was 12 weeks after the 0.75-mg diet had started. (An earlier analysis here would probably have shown that the subjects had reached their low plateau in less than 10 weeks.)

Correlation of amounts excreted with amounts of riboflavin ingested

The amounts of riboflavin excreted in 24 hours and in the 4 hours after subcutaneous administration of 1 mg of riboflavin are compared in table 4 with the amounts of riboflavin ingested per day. The figures in this table are group averages obtained after the subjects had been on the designated deficient diet for approximately three months. In all the diets other than the 0.55-mg diet the three-month level of excretion did not differ appreciably from levels obtained later. Table 2

TABLE 4

Comparison of urinary excretion with daily intake of riboflavin. Except for those on the 0.55-mg diet, the data listed represent plateau levels which were reached in 10 weeks or less on the intake designated [1]

NUMBER OF SUBJECTS ON EACH INTAKE	RIBOFLAVIN INTAKE	DURATION OF DIET AT TIME OF ANALYSES [2]	RIBOFLAVIN EXCRETION 24 HOURS		EXCRETION DURING 4 HOURS FOLLOWING SUBCUTANEOUS INJECTION OF 1 MG OF RIBOFLAVIN
			Amount	Per cent of ingested riboflavin	
	mg	*weeks*	*μg*	%	*μg*
15	0.55	15	51 ± 11	9.3	23 ± 15 [3]
11	0.75	12	73 ± 5	9.7	56 ± 34
12	0.85	15	76 ± 38	8.9	58 ± 22
28	1.1	13	97 ± 62	8.8	70 ± 35
39	1.6	100	434 ± 185	26.5	227 ± 146
12	2.15	10–78	714 ± 293	33.2	297 ± 124
13	2.55	2–44	849 ± 258	33.3	298 ± 172
13	3.55	1.5	1714 ± 300 [4]	48.3 [4]	373 ± 90 [4]

[1] At an 0.85-mg intake or higher the figures obtained at 15 weeks remain at the same level for at least two years more. At a 0.75-mg intake most of the subjects remained at the level designated for 6 months, at which time they were supplemented. A few individuals who remained longer on this diet showed no further change, but whether or not their excretions would have decreased further with additional time is not known.

[2] The time of analyses for diets providing 0.75, 0.85 and 1.1 mg of riboflavin represents the first time plateau levels were obtained which were repeated during subsequent months. The results recorded for the diets providing 1.6, 2.15 and 2.55 are averages of voluminous data obtained during the period of time indicated.

[3] After 7 weeks; cf. table 2.

[4] These results were obtained from the same individuals who received the 2.55-mg intake. After 62 weeks on this intake, their supplementation was further increased to provide a 3.55-mg intake. The values for the 24- and 4-hour excretion periods were obtained one and one-half and three weeks, respectively, after the change in supplementation.

shows that there was a drop to 37 ± 6 μg per day after 27 weeks on this diet, and a gradual continued decrease during the following 6 months to 27 ± 7 μg. Table 3 shows a similar prolonged period of decreasing excretion for the 4-hour load tests.

When the average figures for urinary excretion after three months of a specific dietary regimen (table 4) are converted to per cent of ingested riboflavin, the results indicate a marked difference in the per cent excreted on diets providing 1.1 and 1.6 mg of riboflavin per day. Almost three times as much is excreted at the higher level.

Correlation of urinary excretions of riboflavin with clinical signs of riboflavin deficiency

The first observed sign of possible riboflavin deficiency (angular stomatitis) appeared in subject A1 in September 1947, 4 months after the beginning of the 0.55-mg diet. At this time the 24-hour excretion was between 35 and 40 μg. In no case did any clinical symptoms of riboflavin deficiency appear in subjects who were excreting more than 40 μg in 24 hours. Actually, no other skin lesion appeared until after January 1948, at which time all but three of the subjects (see table 2) were excreting less than 40 μg per day. The details of the clinical examinations will be reported elsewhere.

DISCUSSION

The subjects of these experiments lived in a protected environment relatively free from many irritations and traumas normally encountered in a competitive society. Data on riboflavin requirements obtained under these conditions would therefore provide an estimate of minimum needs only.

The amount of riboflavin excreted in the urine during a 24-hour period has been found to be related to the riboflavin content of the diet and to the length of time the diet is consumed. From the first to the 15th week of observation of subjects receiving 0.55 mg of riboflavin, 9.3% of the ingested

riboflavin was excreted. Between the 15th and 37th week of this dietary regimen, the per cent of ingested riboflavin excreted dropped to 6.7%. There were no significant changes in the following 27 weeks. At dietary levels of 0.75 to 1.1 mg of riboflavin per day, the riboflavin excretion after three months was already at the minmum level of 9% and did not decrease in subsequent months. At levels of from 1.6 to 2.55 mg of riboflavin, approximately 30% of the dietary riboflavin appeared in the urine. Further increase of the riboflavin level of the diet to 3.55 mg per day raised the urinary output to approximately 50% of the amount ingested. These values are in general agreement with those reviewed by Parsons ('44) and by Brewer, Porter, Ingalls and Ohlson ('46).

The fact that a group of 22 subjects were maintained for a period of over two years on diets containing 0.75 to 0.85 mg of riboflavin per day with only one member of the group exhibiting any symptoms that might be attributed to riboflavin deficiency, whereas ariboflavinosis symptoms appeared as early as the 4th month in subjects receiving 0.55 mg of riboflavin per day, makes the excretions at these two dietary levels of particular interest. At some point between these two levels of excretion, 40 and 70 μg of riboflavin per day, one would expect symptoms of ariboflavinosis to appear within 4 to 8 months. Subjects receiving 1.6 mg of riboflavin per day excreted 4 times as much riboflavin as did those receiving 1.1 mg. In terms of per cent excreted of the amount ingested, those receiving the larger amount of riboflavin excreted three times as much as those receiving the smaller amount. From this it would appear that in order for tissues to store riboflavin, dietary levels of more than 1.1 mg per day must be supplied. Unfortunately, the data available do not give information on riboflavin intakes between 1.1 and 1.6 mg per day. However, they make it apparent that under the conditions of this study a level of 1.6 mg per day is adequate for the average individual.

The urinary excretion of riboflavin during the 4 hours following subcutaneous administration of 1 mg of riboflavin parallels the 24-hour excretion. Subjects on the diet which

supplied 0.55 mg of riboflavin per day, during which regime skin lesions appeared in from 4 to 8 months, excreted an average of 19 μg of riboflavin during this 4-hour period. The persons receiving diets containing 0.75, 0.85 and 1.1 mg of riboflavin per day had 4-hour excretions of 56 to 81 μg of riboflavin. Here, too, those individuals receiving the diet containing 1.6 mg of riboflavin showed a marked increase in excretion; the 4-hour riboflavin excretion of this group amounted to 235 μg.

There are important individual variations which should be considered whenever average dietary requirements are discussed. Just as a single rat in a colony may develop skin lesions which are spectacularly reversible by riboflavin at levels which do not similarly affect his siblings, so an occasional human is found who needs more riboflavin than any so-called "requirement" takes into account. Though we did not have signs of ariboflavinosis in the control groups, the large variations in the amount of riboflavin excreted by some of these subjects highlighted this situation. Note subject B12 (table 2), who, on an intake of 2.55 mg per day, consistently excreted less than the average of his group.

Because individual variations in urinary excretion are likely to be large during the early stages of riboflavin depletion, it is not possible to designate definite excretion levels associated with ariboflavinosis. However, under the conditions of this study, in which the subjects were exposed to little or no trauma or irritation, any 24-hour excretion of less than 100 μg can be taken as indicative of a recent dietary regimen providing less than the minimum requirement of riboflavin, and excretions below 50 μg a day as strong evidence that the individual has been on a diet deficient in riboflavin for some time. On the latter level, clinical signs of riboflavin deficiency are to be expected. A diet low in riboflavin, with consequent small urinary excretion of riboflavin, does not *ipso facto* guarantee the development of symptoms of ariboflavinosis. Environmental stresses leading to the need for increased tissue growth and other factors involved in individual variations will remain of

primary importance (Horwitt, Hills, Harvey, Liebert and Steinberg, '49).

The amount of urine excreted is ordinarily a factor to be considered when evaluating the amounts of riboflavin excreted (Feder, Lewis and Alden, '44). However, the direct relationship between urine volume and riboflavin excreted is not apparent when the amounts of riboflavin ingested are small. (See tables 2 and 3.) Therefore, when the subject is on a depleted diet, the total amount of riboflavin excreted gives more information than does the calculation of riboflavin per milliliter of urine.

SUMMARY

Studies of urinary excretion were made at levels of dietary intake of 0.55, 0.75, 0.85, 1.1, 1.6, 2.15, 2.55 and 3.55 mg per day. Data were recorded on 24-hour urine samples and on samples obtained during the 4 hours after subcutaneous administration of 1 mg of riboflavin. The results obtained by both techniques parallel each other.

Subjects exposed to little or no trauma or irritation and receiving 1.6 mg of riboflavin per day excreted 4 times as much riboflavin as those on a 1.1-mg intake. This suggests that the critical riboflavin requirement for an adult male subsisting on 2,200 calories is somewhere between 1.1 and 1.6 mg of riboflavin per day.

Healing of the skin lesions characteristic of ariboflavinosis is retarded when the 24-hour urinary excretion of riboflavin is less than 50 μg. Such a level may be reached in less than 4 months on a diet containing 0.55 mg of riboflavin per day.

A subject depleted in riboflavin can satisfactorily assimilate at least 6 mg of riboflavin at one time. This was borne out by results which indicated that three 2-mg doses at 4-hour intervals were not more effective in repleting a deficient subject than a single dose of 6 mg of riboflavin in 24 hours.

A period of approximately 15 days is required to saturate riboflavin-depleted subjects when a daily supplement of 6 mg of riboflavin is administered.

LITERATURE CITED

BREWER, W., T. PORTER, R. INGALLS AND M. A. OHLSON 1946 The urinary excretion of riboflavin by college women. J. Nutrition, *32:* 583.

FEDER, V. H., G. T. LEWIS AND H. S. ALDEN 1944 Studies on the urinary excretion of riboflavin. Ibid., *27:* 347.

HORWITT, M. K., O. W. HILLS, C. C. HARVEY, E. LIEBERT AND D. L. STEINBERG 1949 Effects of dietary depletion of riboflavin. Ibid., *39:* 357.

HORWITT, M. K., E. LIEBERT, O. KREISLER AND P. WITTMAN 1948 Investigations of human requirements for B complex vitamins. Bull. Natl. Res. Council, no. 116.

HORWITT, M. K., G. SAMPSON, O. W. HILLS AND D. L. STEINBERG 1949 Dietary management in a study of riboflavin requirements. J. Am. Diet. Assn., *25:* 591.

ISBELL, H., J. G. WOOLEY AND H. F. FRASER 1941 The inhibiting effect of urea on the microbiological assay of riboflavin. U. S. Pub. Health Rep., *56:* 282.

PARSONS, H. T. 1944 Further studies on human requirements for riboflavin. Fed. Proc., *3:* 162.

ROBERTS, E. C., AND E. E. SNELL 1946 An improved medium for microbiological assays with *Lactobacillus casei*. J. Biol. Chem., *163:* 499.

SLATER, E. C., AND D. B. MORELL 1946 A modification of the fluorometric method of determining riboflavin in biological materials. Biochem. J., *40:* 644.

SNELL, E. E., AND F. M. STRONG 1939 A microbiological assay for riboflavin. Ind. Eng. Chem., Anal. Ed., *11:* 346.

WILLIAMS, R. D., H. L. MASON, P. L. CUSICK AND R. M. WILDER 1943 Observations on induced riboflavin deficiency and the riboflavin requirements of man. J. Nutrition, *25:* 361.

YEAST IN THE PRODUCTION OF DIETARY MASSIVE HEPATIC NECROSIS IN RATS [1]

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Deficiency of sulfur-containing amino acids (cystine, methionine) and of tocopherol has been recognized recently as the leading dietary condition in the development of massive or zonal hepatic necrosis in rats (György and Goldblatt, '49). It has been found that, regarding hepatic necrosis, the presence of tocopherol may compensate for the absence of sulfur-containing amino acids in the diet, and vice versa.

In our earlier experiments (György and Goldblatt, '49), the incidence of acute necrosis was never higher than 40 to 50%, regardless of whether the reduction in the supply of sulfur-containing amino acids was achieved by the use of a ration low in casein or of one containing yeast as the sole source of protein. In both instances the intake of tocopherol was greatly restricted. These observations were in contrast to the findings

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Preliminary reports were given by one of us (P.G.) at the Third Conference on Biological Antioxidants in 1948 and at the Eighth Conference on Liver Injury in 1949, both conferences sponsored by the Josiah Macy, Jr., Foundation, New York (see respective Transactions).

of Hock and Fink ('43), of Himsworth and his associates (Himsworth, '47; Himsworth and Glynn, '44; Glynn and Himsworth, '44), and of Schwarz ('48), who have reported almost 100% incidence of massive necrosis in rats fed tocopherol-free rations containing yeast as the sole source of protein. The question arose of whether these discrepancies in findings might be explained by inherent differences in the yeast samples used in the various laboratories.

Through the kindness of Professor H. P. Himsworth (London), we were able to obtain the same type of yeast which he used. The following report summarizes our observations on this British yeast as compared with the American type of yeast previously employed by us in our studies on dietary hepatic necrosis in rats.

EXPERIMENTAL METHODS AND FINDINGS

Animal assays

On the recommendation of Professor Himsworth [2] the following "necrogenic" experimental diet was used: yeast 18; corn starch 79; salt mixture (U.S.P. II) 3%. One-half milliliter of peanut oil and two drops of cod liver oil were added to 8 gm of the dry mixture just before feeding. All animals received daily supplements of 20 μg of thiamine, 25 μg of riboflavin, 20 μg of pyridoxine, and 100 μg of calcium pantothenate dissolved together in 1 ml of water. The experimental ration (Y5H), given to 41 rats, contained the British type of bakers' yeast.[3] The control group, comprising 28 rats, received American yeast (diet Y5S).[4] Male rats of the Sprague-Dawley strain were used. The average weight of the rats in the various subgroups at the start of the experiment was 70 to 75 gm. All rats were kept in single cages, with raised, wide-meshed screen bottoms. The daily food intake was measured.

[2] Personal communication.

[3] Obtained from the United Yeast Co., Ltd., London, and later under the designation "D. C. L. Vit. B, yeast" from Distiller's Co., Ltd., Glasgow.

[4] Type 50 B brewers' yeast, Standard Brands, Inc.

There were three subgroups for each of the rations (Y5S and Y5H) used (see table 1). Ration Y5S was given to 14 rats, without special supplement or treatment. In two subgroups, with 7 animals in each, the animals were exposed to "stress" for the purpose of promoting or accelerating the development of acute hepatic necrosis. As "stress" we used in one sub-

TABLE 1

Incidence of hepatic necrosis and other pathologic changes in rats receiving "American" or "British" yeast

CATEGORY OF INTEREST		DIET Y-5-S WITH "AMERICAN" YEAST			DIET Y-5-H WITH "BRITISH" YEAST		
		No supplement	Exercise [1]	Cold exposure [2]	No supplement	Plus tocopherol [3]	Plus cystine [4]
Number of rats		14	7	7	19	11	11
Days on experiment		200	200	200	60–200	200	200
Weight (av.) (gm)	Initial	72	75	70	74	71	73
	Final	141	120	121	95	122	161
Food intake (av.) (gm/day)		7.3	7.5	7.6	6.5	7.1	7.6
Liver	Necrosis	0	0	0	18	0	0
	Normal	14	7	7	1	11	11
Testes	Hypoplasia	3	6	6	3	3	1
	Normal	10	1	1	12	8	9
Stomach	Squamous hypoplasia, keratosis and chronic gastritis [5] of forestomach	13	1	1	..	8	2 (5)*
	Normal	1	6	6	.	2	9 (6)

[1] Exercise in a rotating squirrel cage 4 hr./day from the 50th day.

[2] In cold-room (4–10°C.) 4 hr./day from the 50th day.

[3] Three milligrams/day.

[4] Fifty milligrams/day.

[5] Changes limited to the forestomach.

[6] Includes minor (±) changes. Thus three of the 5 (number within parentheses) rats showed only minor doubtful (±) changes.

group exposure to cold in the cold-room (4 to 10°C.) for 4 hours per day from the 50th to the 200th experimental day. In a second subgroup the experimental animals had to perform exercise in a rotating "squirrel cage" for 4 hours per day, again from the 50th to the 200th experimental day. Ration Y5H, with the British yeast as the sole source of protein, was fed without special supplement to 19 rats. In two further subgroups of 11 animals each, the basal experimental diet was supplemented with either cystine (50 mg per day) or tocopherol (3 mg per day, in the form of natural mixed tocopherols).[5] The experimental period was extended to 200 days, at which time the surviving rats were sacrificed. Macroscopic and microscopic examinations of the liver, kidneys, testes and stomach were performed in all rats.

Acute hepatic necrosis was observed only in the groups receiving the basal ration Y5H, containing the British yeast. Out of 19 rats fed this diet, 16 animals died during the first 150 days; two more animals died between the 150th and 200th experimental day. In the whole group only *one* rat survived the experimental period of 200 days and, at autopsy, it had a normal liver. Thus, for the first time, in our laboratory, acute hepatic necrosis became a regular occurrence in rats fed a diet containing yeast as the sole source of protein.

In all macroscopic and microscopic details the findings confirmed the observations of Himsworth and his associates ('47) on animals kept under identical conditions. Also in accord with Himsworth, animals often showed no premonitory signs of the impending fatal massive hepatic necrosis, and appeared to be in good health until shortly before death. In the few instances when rats were observed during the whole agonal period, no convulsions (Schwarz, '48) occurred and the clinical picture was more that of shock.

In contrast to the necrotic changes in the liver observed in the past (György and Goldblatt, '42, '49) in rats fed experimental rations low in casein, the hepatic necrosis which occurred in rats kept on the (British) yeast ration was, even in

[5] Kindly furnished by Distillation Products Industries, Rochester, N. Y.

the gross, more diffuse, more massive and severely hemorrhagic. In some rats, central and mid-zonal necrosis occurred in some lobes or parts of lobes, while massive necrosis occurred in other lobes or even other parts of the same lobes. In these rats, as in those fed a low casein diet (György and Goldblatt, '42, '49), zonal necrosis never began in the periportal region. This is contrary to the findings of Schwarz. As first pointed out by Himsworth ('47), the necrotic changes were often more pronounced in the left half of the liver than in the right. In this particular group of 19 rats, old scars of healed necrotic foci were not found in the liver.

In all other experimental groups no pathologic changes were found in the livers of the animals. Thus, supplements of cystine or tocopherol prevented the development of hepatic necrosis even in rats receiving the experimental diet containing the British yeast, and substitution of American yeast for the British yeast also deprived the ration of its "necrogenic" quality. Rats fed on the diet containing American yeast and exposed to stress, such as cold or muscular exercise, also failed to develop hepatic necrosis. In the gross, and microscopically, the kidneys of the animals in all experimental groups were normal.

Special attention has been paid to the appearance of the testes at autopsy. In general, the rations used, regardless of whether they contained the American or British yeast, left the testes unimpaired. In this regard, the development of hepatic necrosis by means of the British yeast or its prevention by tocopherol, cystine or the American yeast has been without any appreciable influence.

It is of interest, however, that the forms of stress employed (cold, exercise) in addition to the diet effected significant changes in the testes. These changes consisted of variable reduction in the size of the whole testis as compared with that of a normal rat of the same age and weight, and, microscopically, there were various stages of lack of development of the seminiferous tubules. The least microscopic change observed was failure of development of spermatozoa, and the greatest

alteration seen in these animals was absence of spermatozoa, spermatids and spermatocytes. In none of these animals was the layer of spermatogonia absent, and Sertoli cells were present in all the testes. Although in the tables these changes are referred to as mere hypoplasia, yet this immaturity of the testicular tubules is also implied. In none of these rats was there any atrophy of the testicular tubules or fibrosis of the organ.

Chronic gastritis, squamous hyperplasia, hyperkeratosis, and papillomatous changes, in the forestomach, were regular occurrences in several groups of animals. These findings, when present, were especially impressive in the animals which survived the entire experimental period.

Supplements of cystine reduced slightly the incidence of these gastric changes, whereas supplements of tocopherol were without effect. A significant prevention of these changes in the stomach was achieved by exposure of the animals to stress (cold, exercise). Thus in this respect testes and stomach as target organs behaved in a reciprocal manner: stress, under the conditions chosen, interfered with the development of the testes but improved simultaneously the condition of the stomach.

The regular occurrence of massive hepatic necrosis in rats fed the experimental ration with the British yeast as the sole source of protein, and its absence when American yeast was substituted for the British yeast, raise the question of the difference between these two brands of yeast and the explanation of their different effects on the liver.

The British yeast is a liver bakers' yeast, the American yeast used in these experiments a heat-killed brewers' yeast. The possibility that the British yeast, being an active yeast, may be less well digested and utilized (Price et al., '47) than the inactive American yeast received no direct support in the experiments of Himsworth and Lindan, who found no difference between the necrogenic effects of live and heat-killed

British yeast.[6] The average food intake in the group of rats fed the diet with the American yeast was only slightly higher than in the group fed the British yeast. As a matter of fact, for a comparable period of time (the first 14 weeks), the average daily food intake amounted to 6.5 gm for the group given the American yeast and 6.3 gm for the group that received the British yeast. On the other hand, the gain in weight during the total experimental period was distinctly higher in the group of animals receiving the American yeast as the source

TABLE 2

Chemical analysis of "British" and "American" yeast

ANALYSIS	AMERICAN	BRITISH
	%	%
Nitrogen	7.83	6.76
Total sulfur	0.86	0.65
Methionine [1]	0.79 [3]	0.80 [3]
Cystine [2]	0.43 [4] ± 0.06	0.72 [5] ± 0.05

[1] Microbiological assay also showed little difference in the methionine content of the two yeasts; the values were slightly lower than those obtained by the chemical method.

[2] Checks on aliquots of the same sample were not wholly satisfactory; the results in the table are the averages of 9 determinations with the average deviation.

[3] Equivalent to 0.17% S.

[4] Equivalent to 0.12% S.

[5] Equivalent to 0.19% S.

of protein. The possibility that the better growth-promotion, as well as the suppression of hepatic necrosis, by the American yeast might be due to a higher content of the sulfur-containing amino acids (cystine, methionine) required a direct answer through chemical analysis of both brands of yeast.

Nitrogen was determined by the micro-Kjeldahl procedure, total sulfur by the method of Pirie ('32), cystine by the β-naphthoquinone-4 sulfonate method (Csonka et al., '44), and methionine by the method of Lavine ('43). Cystine and methionine determinations were carried out on samples that had

[6] Personal communication from Professor Himsworth.

been hydrolyzed 18 hours with 20% hydrochloric acid and the acid removed by vacuum distillation. The results obtained are summarized in table 2.

The higher values for total N and total S in the American yeast compared with those in the British yeast are noteworthy. Of the sulfur-containing amino acids, the British yeast contained slightly more cystine than the American yeast. The values obtained for methionine as well as the unexplained excess of sulfur in these two brands of yeast are in good accord with similar findings in the literature (Goyco and Asenjo, '49; Schwarz, '49).

Chemical isolation procedures do not indicate the presence of tocopherol in yeast.[7] The possibility, although remote, that yeast may contain tocopherol or a tocopherol-like substance which cannot be extracted through the usual chemical procedures was considered and investigated by a new technique. It has been shown recently (Rose and György, '49) that in rats previously kept on a mixed grain diet, beginning vitamin E deficiency may be demonstrated in three to 7 days after they are put on a diet free of vitamin E, with or without fat, and consisting mainly of casein, sugar, salt mixture and vitamins. The red blood cells of such rats, washed and suspended in a buffered saline solution, will be hemolyzed by minute amounts of dialuric acid, and less readily by ascorbic acid or cystine. This hemolysis may be prevented by tocopherol, added *in vitro* to the assay mixture, and will not occur when red cells originate in rats receiving tocopherol in their diet. Rats put on our usual necrogenic (hepatic) semi-synthetic rations containing American and British yeast, respectively, have shown after three days on the experimental diet this peculiar fragility of the red blood cells, manifesting itself by hemolysis under the influence of dialuric acid. Thus, both brands of yeast appear to be essentially free from tocopherol.

[7] Personal communication from Dr. P. L. Harris, Distillation Products Industries, Rochester, N. Y.

DISCUSSION

With regard to the production of acute hepatic necrosis in rats by dietary means the study here reported permits three definite conclusions:

1. Acute massive hepatic necrosis may be produced with great regularity and with an incidence of 90% and more in rats fed a semi-synthetic diet with a special British brand of yeast as sole source of protein. Acute massive hepatic necrosis did not occur when an American brand of yeast was used.

2. The British yeast diet when supplemented with cystine or tocopherol loses its necrogenic quality.

3. The analytical data obtained with respect to sulfur-containing amino acids (cystine and methionine) and tocopherol in the British and American brands of yeast used give no explanation for the difference in their necrogenic activity.

The slightly higher food intake and better growth curve of rats fed the ration with the American yeast than of animals kept on the British yeast diet do not appear to offer an explanation for the absence of hepatic necrosis in the first group and its regular occurrence in the other. In this connection it should also be pointed out that Hock and Fink ('47) have found no appreciable difference in the necrogenic quality of various strains of yeast (brewers' or bakers' yeast or torula) regardless of their varying effect on the growth of experimental animals when used in rations as the source of protein.

Not only its high incidence but also its truly massive and hemorrhagic character distinguishes the hemorrhagic necrotic change seen in rats fed the British yeast ration from that observed in the past (György and Goldblatt, '49) in rats kept on rations low in casein (cystine, methionine) and tocopherol.

Schwarz ('48, '49) speaks of "rat-eclampsia," bringing under this heading not only the massive hepatic necrosis developed in rats on a yeast diet but also the pre-agonal convulsions and some renal changes ("glomerulo-nephrosis"). Schwarz also points out that the necrotic changes which develop in the livers of rats on a yeast diet seem to start in the periphery and not — as in other forms of experimental dietary

hepatic necrosis — in the central zone of the lobule. Apart from the fact that we observed pre-agonal convulsions only rarely in our experimental animals, our findings with respect to the liver and the kidneys do not support the analogy with "eclampsia" in its broader sense.

As stated before (György and Goldblatt, '49), it is difficult to reconcile pure deficiency as the possible cause of dietary hepatic necrosis with the interchangeability in its prevention of substances as different chemically as the sulfur-containing amino acids and tocopherol. It is possible (György and Goldblatt, '49; György, '49) that this interchangeability of cystine or methionine and tocopherol is due, primarily, more to an underlying detoxifying mechanism than to the correction of a deficiency. The sulfur-containing amino acids, as well as vitamin E, are known as detoxifying agents in counteracting the noxious effect of hepatotoxic substances and related poisons (György and Goldblatt, '49). As detoxifying agents, vitamin E and cystine may act through the intermediary of some oxido-reductive reactions, although the possibility of different points of attack resulting in the same effect, i.e., in the prevention of hepatic necrosis, cannot be excluded.

The analytical data with respect to the sulfur-containing amino acids and tocopherol do not explain the difference in the "necrogenic" activity of the British and American brands of yeast. Thus, the fact that the British yeast appeared to be particularly conducive to the development of acute massive hepatic necrosis may be due not only to the lack of protective (detoxifying) factors (cystine, methionine, vitamin E) but perhaps also to the presence of some unidentified toxic factors in the yeast; or, more probably, to the directly or indirectly supported production of some toxic metabolites. Such toxic substances may originate in the intermediary metabolism or under the influence of the intestinal flora in the intestinal tract; in particular, in the large intestine. In any case, British yeast may promote the production of such toxic factors.

As was first pointed out by Himsworth ('47), the necrotic changes in the liver seem to prefer the left lobe. He saw in this

difference circumstantial evidence for the so-called trophopathic (dietary deficiency) etiology of the condition. Portal vein blood from the small intestine supplies mainly the right side of the liver, and from the large intestine (including stomach and spleen) the left half of the liver. Protective food constituents — cystine and methionine, as well as vitamin E — are absorbed from the small intestine and directed to the right side of the liver. Blood from the large intestine must be low in, or free from, such protective food factors, and, in consequence, the deficiency condition — necrosis — should be more pronounced in the left liver. Also, if toxic products originate in the large intestine, the left liver should show more signs of injury than the right liver.

All these are, of course, purely hypothetical considerations. In this connection, however, it should be borne in mind that hepatic necrosis due to simple dietary deficiency is unknown in clinical medicine. The interplay of toxic factors tallies much better with clinical observations as far as hepatic necrosis is concerned. Clinically, necrosis associated with viral hepatitis is the most common form of this acute hepatic injury. Various chemical poisons, such as halogenated hydrocarbons, phosphorus, drugs, and unidentified "cryptogenic poisons" such as those postulated in eclampsia, are other common etiological factors in the development of hepatic necrosis in man.

The necrogenic quality of the British yeast and its absence in the American yeast require further elucidation. In this connection, it is of further interest that the gain in weight throughout the whole experimental period was better in the group of animals receiving the diet with the American yeast, compared not only with the group of animals fed the basal ration with the British yeast but also with the animals which received the British yeast diet supplemented with vitamin E, and which remained free from hepatic necrosis. The analytical data for the sulfur-containing amino acids in these two brands of yeast offer no clue to the differences in weight curves.

In our experiments, young rats of known strain (Sprague-Dawley) and of known previous dietary history were used.

Vitamin E is stored for a long time in the body. Thus it is not surprising that in older rats, with their well established stocks of vitamin E, or even in younger rats fed prior to the experiment a diet particularly rich in vitamin E, the production of hepatic necrosis may be long delayed (Himsworth and Lindan, '49).

Gastric changes (hyperplasia, hyperkeratosis, ulcero-papillomatous lesions, and chronic inflammation) were a common and prominent occurrence in the forestomachs of groups of rats receiving the basal rations, regardless of whether the British or the American brand of yeast was used. In some the hyperplasia was even atypical, but definite malignant change was not observed. Such pathologic alterations in the lining of the rumen have been observed by others (Berg, '42; Sugiura, '42) in rats subjected to a great variety of experimental conditions, prominent among which were starvation, dietary deficiency (protein, various vitamins), carcinogenic agents, and local irritants.

Supplementation with vitamin E was without effect on the gastric changes, whereas cystine, when added to the British yeast ration, brought about a slight but definite improvement in the condition of the gastric lining. It was surprising to find that in rats exposed to "stress," such as cold or muscular exercise, a statistically significant reduction in the incidence of the gastric lesions became apparent. This was achieved without any alteration in the composition of the diet. This finding, especially when considered in relation to the great variety of etiologic conditions found responsible for these pathologic changes in the forestomach of rats, raises the question of a possible adrenal activity in the production and prevention of the gastric lesions. The reciprocal relation of gastric and testicular changes in our rats exposed to "stress" may indicate an intimate interrelation existing between testicular and adrenal activity.

SUMMARY

In rats fed a semi-synthetic ration with a British brand of bakers' yeast as the sole source of protein massive hepatic

necrosis occurred with great regularity (more than 90%). Substitution of an American brand of brewers' yeast for the British yeast deprived the diet of its "necrogenic" quality.

Supplements of cystine or of vitamin E were equally effective in the prevention of the dietary hepatic necrosis which develops in rats on a diet containing British yeast as the sole source of protein.

The necrogenic activity of the British yeast could not be explained by a difference in the content of the sulfur-containing amino acids (cystine, methionine) or of vitamin E in the British and American brands of yeast.

The possibility has been discussed that so-called dietary hepatic necrosis is due more to some unidentified "toxic" factors than to pure deficiency. Tocopherol and cystine may act as "detoxifying" agents.

Lesions consisting of squamous hyperplasia, hyperkeratosis, ulcers, and chronic inflammation were common in the forestomachs of rats receiving the basal yeast ration, with or without supplementation with vitamin E. Addition of cystine to the diet and, more definitely, exposure to "stress" (cold, muscular exercise), without any change in the dietary regime, brought about a reduction in the incidence of these gastric lesions.

Testicular hypoplasia was observed in animals exposed to "stress" but was uncommon in all other groups. On the other hand, the pathological changes in the forestomach occurred less commonly and were not as pronounced in the rats subjected to "stress."

LITERATURE CITED

Berg, B. N. 1942 Pathological changes in nutritional gastritis in rats. Am. J. Path., *18:* 49.

Csonka, F. A., H. Lichtenstein and C. A. Denton 1944 Cystine determination in proteins and foods. J. Biol. Chem., *156:* 571.

Glynn, L. E., and H. P. Himsworth 1944 Massive acute necrosis of liver; its significance and experimental production. J. Path. Bact., *56:* 297.

Goyco, J. A., and C. F. Asenjo 1949 Net protein and growth-promoting values of three different types of yeast prepared under identical conditions. J. Nutrition, *38:* 517.

GYÖRGY, P. 1949 Liver Injury. Trans. 8th Conf., Josiah Macy, Jr., Foundation, New York, N. Y.

GYÖRGY, P., AND H. GOLDBLATT 1942 Observations on the conditions of dietary hepatic injury (necrosis, cirrhosis) in rats. J. Exp. Med., *75:* 355.

———— 1949 Further observations on the production and prevention of dietary hepatic injury in rats. Ibid., *89:* 245.

HIMSWORTH, H. P. 1947 The Liver and its Diseases. Oxford, Blackwell and Co., Ltd.

HIMSWORTH, H. P., AND L. E. GLYNN 1944 Prevention of experimental massive hepatic necrosis by methionine. Clin. Sci., *5:* 133.

HIMSWORTH, H. P., AND O. LINDAN 1949 Dietetic necrosis of the liver: the influence of α-tocopherol. Nature, *163:* 30.

HOCK, A., AND H. FINK 1943 Über den biologischen Ergänzungswert verschiedener Nahrungsproteine; über die Bedeutung des Cystins für den Stoffwechsel, zugleich ein Beitrag zur Verbesserung des Hefeeiweisses. Zschr. phys. Chem., *279:* 187.

———— 1947 Prevention of nutritional injury of the liver in rats by l-cystine. Zschr. Naturforschg., *2b:* 203. Through Chem. Abstr., *42:* 3470e, 1948.

LAVINE, T. F. 1943 An iodometric determination of methionine. J. Biol. Chem., *151:* 281.

PIRIE, N. W. 1932 Studies in the sulphur metabolism of the dog: the metabolism of methionine and related sulphides. Biochem. J., *26:* 2041.

PRICE, E. L., M. M. MARQUETTE AND H. T. PARSONS 1947 Availability of vitamins from yeasts; availability to human subjects of riboflavin from fresh and dried bakers' yeasts varying in viability. J. Nutrition, *34:* 311.

ROSE, C. S., AND P. GYÖRGY 1949 Hemolytic action of alloxan and alloxan derivatives. Fed. Proc., *8:* 244.

SCHWARZ, K. 1948 Die Ratten-eklampsie, eine Ambogene. Zschr. phys. Chem., *283:* 186.

———— 1949 Liver Injury. Trans. 8th Conf., Josiah Macy, Jr., Foundation, New York, N. Y.

SUGIURA, K. 1942 Relation of diet to development of gastric lesions in rat. Cancer Res., *2:* 770.

PANTOTHENIC ACID STUDIES

IX. THE INFLUENCE OF DIETARY PANTOTHENIC ACID UPON A PANTOTHENIC ACID CONJUGATE (PAC) IN RAT TISSUES [1,2]

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ONE FIGURE

(Received for publication January 18, 1950)

Previous studies in this laboratory have revealed the presence in tissues of a non-dialyzable conjugated form of pantothenic acid (PAC), which also contains glutamic acid and is active for the growth of *Acetobacter suboxydans* (King, Locher and Cheldelin, '48; King, Fels and Cheldelin, '49). It appears to be a different entity from coenzyme A or the other conjugates of pantothenic acid which have been described during the past several years (Lipmann, Kaplan,

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Novelli, Tuttle and Guirard, '47; Neal and Strong, '43; Wright, '43). A major physiological role for coenzyme A has been established by Lipmann and Ochoa and their coworkers in its association with the metabolism of acetate (Lipmann and Kaplan, '46; Novelli and Lipmann, '47; Stern and Ochoa, '49). Although similar properties have not been demonstrated for PAC, it nevertheless appears to be a functional derivative of the vitamin on the basis of the discovery (King and Cheldelin, '50; King, Stearman and Cheldelin, '48) that it is more active than free pantothenic acid in several species of bacteria, and is superior to pantothenic acid in counteracting a growth inhibitor of *A. suboxydans* which interferes with the utilization of this vitamin.

Further information regarding the essential nature of PAC has been sought in the present work by determining its retention in the tissues of pantothenic acid-deficient rats. The evidence obtained, described below, indicates that as rats are depleted of pantothenic acid PAC, as well as other conjugates, is retained to a relatively greater degree than is the free vitamin. PAC has been found to constitute a significant portion of the total pantothenic acid in rat tissues, and it seems probable that this conjugate represents an important form of the vitamin for the rat.

EXPERIMENTAL

In the present study, three experiments were performed. The first two were devoted to the effect of dietary pantothenic acid upon the *A. suboxydans* activity of various rat tissues. It was assumed at first that this organism would measure only PAC; however, a sample of coenzyme A (generously supplied by Dr. Lipmann) was also found active for *A. suboxydans,* and more recently Novelli, Flynn and Lipman ('49) reported the fact that coenzyme A is active for this microorganism. For this reason a second experiment was performed with more animals to test the *A. suboxydans* activity and the acetylating power of tissue preparations which had been dialyzed to remove coenzyme A. Finally,

a third experiment was carried out to determine the possible effect of a dietary glutamic acid deficiency upon PAC formation by the rat.

The animals in the first experiment consisted of one litter of 9 28-day-old rats of the Evans-Long strain. They were

TABLE 1

The composition of the basal diets

INGREDIENTS	AMOUNTS	
	Experiments 1 and 3	Experiment 2
Cerelose (gm)	730	720
Casein, vitamin-free (gm)	180 [1]	180
Sulfaguanidine (gm)		10
Cod liver oil (gm)	10	10
Mazola (gm)	50	50
Salt mixture (gm)	40	40
Menadione (mg)		50
Choline chloride (mg)		5,000
Inositol (mg)		2,000
Thiamine (mg)	20	40
Riboflavin (mg)	20	80
Niacin (mg)		500
Pyridoxine hydrochloride (mg)	40	40
Biotin (mg)		0.2
p-Aminobenzoic acid (mg)		250
Folic acid (mg)		10
Calcium pantothenate (mg)	200 [2]	200 [2]

[1] A mixture of purified amino acids was used in place of casein in experiment 3. Its composition was as follows (amount in grams per kilogram of diet): glycine 1.0, DL-alanine 7.0, DL-serine 2.0, DL-valine 20.0, DL-leucine 24.0, DL-isoleucine 16.0, L-cystine 2.0, DL-methionine 8.0, DL-threonine 14.0, DL-tryptophan 4.0, L-aspartic acid 2.0, L-glutamic acid 20.0 (used only in the control diet), DL-lysine 30.0, L-proline 2.0, L-histidine 9.5, L-arginine 6.0, L-tyrosine 5.0, L-phenylalanine 5.0, and sodium bicarbonate 17.5.

[2] Included only in the diet of the control rats and the rats in experiment 3.

kept in individual wire cages and allowed water and food ad libitum. Four animals were used as controls and the remainder were made pantothenic acid-deficient. The diet listed in table 1 was fed to the experimental animals, whereas the controls received in addition 200 mg of calcium panto-

thenate per kilogram of diet. After 61 days on the above regimens the animals were sacrificed by a blow on the head. Immediately after expiration, they were dissected and the brain, skeletal muscle, heart, kidney and liver were rapidly removed. They were freed from blood and connective tissue as much as possible and were then stored briefly in a refrigerator until the dissections were completed. The pooled samples of each tissue were masticated in a Potter-Elvehjem microhomogenizer or in a Waring Blendor to a fine pulp with an adequate amount of water and then diluted to the desired volume. The suspensions were steamed for 5 minutes, cooled immediately and then filtered. The filtrates were used as the stock samples for analysis.

In the second experiment the animals consisted of 17 rats from different litters. Their body weights at the beginning of the experiment averaged 93 gm for the males and 86 gm for the females in both the control and experimental groups. One per cent of sulfaguanidine was incorporated in the ration in an effort to reduce bacterial synthesis of the vitamin in the intestine. After a preliminary three-week depletion period, during which no B vitamins were given to either group, the levels of all B vitamins except pantothenic acid were raised to the amounts indicated in table 1. During 4 additional weeks on the pantothenic acid-free diets, the deficient animals developed bloody whiskers and "whisky noses" and their fur became dull in appearance. At the end of this time their average weight was 89 gm compared to 116 gm for the controls. They were sacrificed and their tissues were prepared for analysis as described above except that they were frozen in cracked dry ice immediately after removal from the body.

Dialysis of the stock samples from experiment 2 was carried out against running distilled water at temperatures below 15°C. The acetylation of sulfanilamide was performed with fresh dialyzed pigeon liver as the apoenzyme. This procedure, as well as the testing of *A. suboxydans* activity and the determination of free pantothenic acid, was the same

as that described in a previous paper (King, Fels and Cheldelin, '49).

In the third experiment, which dealt with dietary glutamic acid deficiency and conjugate activity, 6 animals from a single litter were used. Of these, two were used as controls and 4 as the "glutamic acid-deficient" animals. The diet was the same as in the first experiment except that the casein was replaced by a mixture of pure amino acids. The feeding was continued for 55 days. Tissue samples were prepared as in experiment 1.

RESULTS AND DISCUSSION

Pantothenic acid deficiency

Growth curves. Curves 1 and 2 of figure 1 depict the average gain in weight made by the animals in the first experiment. It will be noted that the pantothenic acid-deficient rats, although smaller than the controls, were still gaining weight steadily at the end of the 61-day period. It is possible that this may have been due to intestinal synthesis of the vitamin, since the deficient animals which received sulfaguanidine in experiment 2 barely recovered their starting body weights after losing approximately 10 gm during the preliminary depletion period. Those receiving pantothenic acid gained approximately 25 to 30 gm over their starting weights.

The effect of takadiastase and papain upon release of bound pantothenic acid. It was suggested editorially (Anonymous, '47), and confirmed experimentally (Novelli, Kaplan and Lipmann, '49; Nielands and Strong, '48), that the conventional treatment of tissue samples with takadiastase and papain (Cheldelin, Eppright, Snell and Guirard, '42) is not adequate for complete liberation of pantothenic acid. The data in the first two groups of table 2 bear this out, for it is evident that in the boiled extracts used in the present work (in which the autolytic enzymes had been destroyed), takadiastase and papain released only a relatively small pro-

portion of the total pantothenic acid which was available to *A. suboxydans*. Release of the vitamin from PAC, as well as from coenzyme A, has been accomplished in this laboratory through the combined use of alkaline phosphatase and a chicken liver enzyme (Nielands and Strong, '48) and

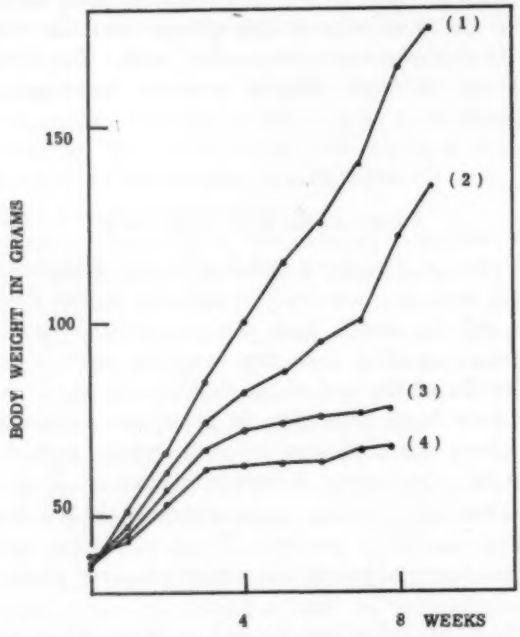

Fig. 1 Growth curves of animals in experiments 1 and 3. (1) Pantothenic acid control; (2) pantothenic acid-deficient; (3) glutamic acid control; (4) glutamic acid-deficient.

it seems probable that essentially all of the pantothenic acid present in tissues is released by this combination of enzymes.

Free and bound vitamin during depletion. The data in table 2 confirm the observation by Novelli, Kaplan and Lipmann ('49) that the bulk of the pantothenic acid in tissues is in bound form. Although the degree of release of the vitamin (and hence its apparent presence in free form) de-

pends in large part upon the opportunity for autolysis of the tissues, this factor may probably be ignored in the present studies. It may be calculated on the basis of the figures presented that only about 3% of the vitamin was free in liver tissue in the control animals. Although the proportion of free vitamin is higher in the other tissues studied, the highest figure, found in heart muscle, was only 16%. Moreover, during depletion, the proportion of free pantothenic

TABLE 2

Levels of free and combined pantothenic acid in tissues of deficient and control rats

	FREE PANTOTHENIC ACID [1]			PANTOTHENIC ACID AFTER TAKADIASTASE AND PAPAIN DIGESTION [2]			A. SUBOXYDANS ACTIVITY		
	Deficient	Control	Relative activity	Deficient	Control	Relative activity	Deficient	Control	Relative activity
	μg/gm	μg/gm	%	μg/gm	μg/gm	%	mg/gm [3]	mg/gm [3]	%
Liver	2.0	3.8	53	2.4	5.6	43	17.2	31.3	55
Muscle	0.4	1.0	40	1.9	3.5	54	4.8	8.2	59
Heart	0.8	8	10	1.7	13	13	8.4	12.5	67
Kidney	1.6	10	16	2.1	13	16	17.8	25.6	70
Brain							10.5	14.3	74

[1] Free pantothenic acid = amount of vitamin present in filtered boiled extracts, as determined directly with *L. arabinosus*.

[2] See Cheldelin et al. ('42). Digestion was carried out on boiled extracts.

[3] Milligrams of PAC sample 801 per gram of tissue. Sample 801 contains approximately 4 μg of bound pantothenic acid per milligram. The total concentration of pantothenic acid in liver tissue (control) is thus 125 μg per gram, and so forth.

acid becomes even less (table 2) in all tissues except liver; the relative amounts of bound forms in the deficient animals as compared to the controls range from 55% in liver to 74% in the brain, but the corresponding relative levels of the free vitamin range from 53% in the liver down to 10% in the heart. These observations, taken together with knowledge of the biochemical role of coenzyme A, suggest strongly that in the economy of the animal, free pantothenic acid repre-

TABLE 3

Conjugate activity after dialysis of rat tissues

A. suboxydans activity

HOURS OF DIALYSIS	LIVER			MUSCLE			HEART			KIDNEY		
	Control	Deficient	Relative activity	Control	Deficient	Relative activity	Control	Deficient	Relative activity	Control	Deficient	Relative activity
	mg/gm [1]	mg/gm [1]	%	mg/gm [1]	mg/gm [1]	%	mg/gm [1]	mg/gm [1]	%	mg/gm [1]	mg/gm [1]	%
0	44	17	39	12	8	67	12	6	50	40	22.5	56
2										34	11	32
4	34	12	33									
45				4.8	4.1	85	4.2	3.4	81	8.0	5.5	69
68–70	12	7	58	4.4	4.2	95						
Ratio of non-dialyzable conjugate to total conjugate activity (%)	27	41		37	50		35	57		20	24	

[1] See footnote 3, table 2.

sents a relatively less essential form of the vitamin, perhaps inactive altogether per se, whereas the conjugates are of greater physiological significance.

Since, as was pointed out above, the *A. suboxydans* responses shown in table 3 represent coenzyme A as well as PAC, the values obtained with the experiment 2 animals (table 3) are of special interest. Here it may be seen that although most of the activity for *A. suboxydans* is lost upon

TABLE 4

The acetylation of sulfanilamide by rat tissues

TISSUE		HOURS OF DIALYSIS	SULFANILAMIDE ACETYLATED
			μg
Kidney, (59 mg)	control	0	40
		2	10
		45	0
(59 mg)	deficient	0	12
		4	0–4
Liver, (68 mg)	control	0	56
		4	20
		70	0
(68 mg)	deficient	0	15
		4	4
		70	0

dialysis (varying from 63% in muscle to 80% in the kidneys of the control animals), there is nevertheless a significant residual activity even after 45 to 68 hours of dialysis. This is especially true in the deficient animals, where 24% (kidney) to 57% (heart) is non-dialyzable. That this material cannot represent coenzyme A is indicated by the failure of the dialyzed samples to accomplish acetylation, as shown in table 4. Here, although the power to acetylate sulfanilamide is less in the tissues of the deficient animals than in those

of the controls (this has been demonstrated previously by Olson and Kaplan, '48), it diminishes very rapidly in both control and deficient animals as the tissues are dialyzed. This virtually complete loss of acetylating power after 2 to 4 hours, as contrasted to the more gradual disappearance of *A. suboxydans* activity, suggests that rat tissues may actually contain more than one *dialyzable* pantothenic acid conjugate, in addition to the relatively non-dialyzable PAC.

The suggestion has been made that coenzyme A may be converted to PAC, possibly through degradation (Novelli, Flynn and Lipmann, '49). In view of the fact that PAC is less readily dialyzed than coenzyme A, its formation as a degradation product of coenzyme A seems unlikely, although it is not possible to rest the case on the basis of this single observation. Moreover, although a conversion of some sort might occur in the living animal, it is unlikely that it could take place during dialysis, since the residual PAC contents, particularly in heart and skeletal muscle, were nearly the same in both the deficient and control animals even though before dialysis the levels in the control group were much higher. If conversion of coenzyme A to PAC took place, the final PAC values after dialysis would be expected to parallel the initial values more closely. Novelli, Flynn and Lipmann ('49) report the formation of an enzymatically inactive material from coenzyme A through autolysis. However, the manner of preparation of tissue samples in the present experiments would appear to prevent autolysis, and it seems reasonable to conclude that the PAC contents observed represent material which existed in the rat tissues at the time the animals were sacrificed. PAC may therefore be regarded for the rat as an important form of pantothenic acid, which is conserved by the animal during depletion at the expense of the free vitamin. This is especially true in the heart and skeletal muscle, where, as is stated above, the PAC content is practically as great in deficient animals as in controls.

The present results are in apparent disagreement with the observations of Novelli, Kaplan and Lipmann ('49), who

concluded, on the basis of their assays of bound pantothenic acid in animal tissues together with a measure of the acetylating power of these materials, that virtually all of the pantothenic acid present is represented by coenzyme A. The reasons for this discrepancy are not obvious, but they may be due in part to the existence of coenzyme A-like material in at least two forms (Strong, '49; Cheldelin, Teply and Green, '49). These are claimed to possess considerably different abilities to acetylate sulfanilamide per mole of pantothenic acid (Strong, '49). If these forms should both be found to be present in significant amounts in tissues, it is quite possible that the coexistence with them of non-acetylating forms, such as PAC, might be overlooked.

Dietary glutamic acid deficiency. Curves 3 and 4 of figure 1 demonstrate the growth rate of rats on a diet in which the casein of experiment 1 was replaced by an equal quantity of amino acids. The experimental animals which were deprived of glutamic acid were not fed extra nitrogenous material. Their poorer weight gain may reflect this in part, inasmuch as Lardy and Feldott ('49) and Rose, Smith, Womack and Shane ('49) have shown that non-essential amino acids or ammonium salts can improve weight gains on diets containing threshold amounts of the indispensable amino acids. The inferior performance on the glutamic acid-deficient diet confirms the observations of Rose et al. (Rose, Smith, Womack and Shane, '49; Rose, Oesterling and Womack, '48). Of more direct interest in the present study, however, is the finding that the amounts of both free and combined pantothenic acid in the tissues were essentially the same in both the "glutamic acid-deficient" animals and the group which received large supplements of this amino acid. Evidently the synthesis of glutamic acid by the rat is at all times sufficient to permit the formation of adequate amounts of PAC.

SUMMARY

1. Albino rats on a pantothenic acid-deficient diet developed pronounced deficiency symptoms over an 8- to 9-

week period. Assay of the brain, liver, kidney, heart and skeletal muscle of deficient and control animals revealed that the content of both free and bound pantothenic acid had diminished during this period.

2. Free pantothenic acid, as measured directly by *L. arabinosus*, represented only 3 to 16% of the total pantothenic acid content of the various tissues in control animals; however, the proportion of the free vitamin was even less in the deficient animals. From 47 to 90% of the free vitamin was lost during depletion of the animals, whereas the decreases in the conjugated forms (measured by *A. suboxydans*) ranged from 26 to 45%.

3. A significant portion of the bound pantothenic acid in rat tissues was found to exist in non-dialyzable form. In the controls this proportion ranged from 20 to 37% and in the deficient animals from 24 to 57%. The active principle remaining after dialysis was not active in the acetylation of sulfanilamide, and it was considered that this represented the conjugate (PAC) recently described in this laboratory.

4. It was concluded that PAC may be regarded as an important form of pantothenic acid for the rat.

5. Dietary glutamic acid deficiency has no effect upon the amounts of the free or conjugated forms of pantothenic acid in rat tissues.

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LITERATURE CITED

ANONYMOUS 1947 Present knowledge of pantothenic acid in nutrition. Nutrition Rev., *5:* 291.

CHELDELIN, V. H., M. A. EPPRIGHT, E. E. SNELL AND B. M. GUIRARD 1942 Enzymatic liberation of B vitamins for plant and animal tissues. Univ. of Texas Publ. 4237, p. 15.

CHELDELIN, V. H., L. J. TEPLY AND D. E. GREEN 1949 Unpublished observations.

KING, T. E., AND V. H. CHELDELIN 1950 Pantothenic acid studies. VIII. Growth of microorganisms and counteraction of antimetabolites with a pantothenic acid conjugate (PAC). J. Bact., *59:* 229.

KING, T. E., I. G. FELS AND V. H. CHELDELIN 1949 VI. A biologically active conjugate of pantothenic acid. J. Am. Chem. Soc., *71:* 131.

KING, T. E., L. M. LOCHER AND V. H. CHELDELIN 1948 III. A pantothenic acid conjugate active for *Acetobacter suboxydans.* Arch. Biochem., *17:* 483.

KING, T. E., R. L. STEARMAN AND V. H. CHELDELIN 1948 V. Reversal of 2-chloro-4-aminobenzoic acid inhibition in *E. coli* by pantothenic acid. J. Am. Chem. Soc., *70:* 3969.

LARDY, H. A., AND G. FELDOTT 1949 The net utilization of ammonium nitrogen by the growing rat. J. Biol. Chem., *179:* 509.

LIPMANN, F., AND N. O. KAPLAN 1946 A common factor in the enzymatic acetylation of sulfanilamide and of choline. Ibid., *162:* 743.

LIPMANN, F., N. O. KAPLAN, G. D. NOVELLI, L. C. TUTTLE AND B. M. GUIRARD 1947 Coenzyme for acetylation, a pantothenic acid derivative. Ibid., *167:* 869.

NEAL, A. L., AND F. M. STRONG 1943 Existence of an alkali stable derivative of pantothenic acid in biological materials. J. Am. Chem Soc., *65:* 1659.

NIELANDS, J. B., AND F. M. STRONG 1948 The enzymatic liberation of pantothenic acid. Arch. Biochem., *19:* 287.

NOVELLI, G. D., R. M. FLYNN AND F. LIPMANN 1949 Coenzyme A as growth stimulant for *Acetobacter suboxydans.* J. Biol. Chem., *177:* 493.

NOVELLI, G. D., N. O. KAPLAN AND F. LIPMANN 1949 The liberation of pantothenic acid from coenzyme A. Ibid., *177:* 97.

NOVELLI, G. D., AND F. LIPMANN 1947 The involvement of coenzyme A in acetate oxidation in yeast. Ibid., *171:* 833.

OLSON, R. E., AND N. O. KAPLAN 1948 The effect of pantothenic acid deficiency upon the coenzyme A content and pyruvate utilization of rat and duck tissues. Ibid., *175:* 515.

ROSE, W. C., M. J. OESTERLING AND M. WOMACK 1948 Comparative growth of diets containing 10 and 19 amino acids, with further observations upon the role of glutamic acid and aspartic acid. Ibid., *176:* 753.

ROSE, W. C., L. C. SMITH, M. WOMACK AND M. SHANE 1949 The utilization of the nitrogen of ammonium salts, urea and certain other compounds in the synthesis of non-essential amino acids *in vivo.* Ibid., *181:* 307.

STERN, J. R., AND S. OCHOA 1949 Enzymatic synthesis of citric acid by condensation of acetate and oxalacetate. Ibid., *179:* 491.

STRONG, F. M. 1949 Private communication.

WRIGHT, L. D. 1943 The state of pantothenic acid in blood. J. Biol. Chem., *141:* 261.

THE EFFECT OF DIET ON THE SUSCEPTIBILITY OF RATS TO POISONING BY 2, 4, 6-TRINITROTOLUENE (TNT)[1]

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TWO FIGURES

(Received for publication January 3, 1950)

Stimulated by the problem of the poisoning of industrial workers by trinitrotoluene (TNT) which first arose during World War I, certain investigators have studied experimentally the influence of diet on susceptibility to this compound. Voegtlin, Hooper and Johnson ('20, '21) obtained results suggesting that diet does influence the toxicity of TNT in dogs, since there were less anemia and longer survival on a bread, milk and meat diet and on meat alone than on a diet of milk and bread. Himsworth and Glynn ('42) reported that rats on a high fat diet developed severe symptoms and liver damage, while those on high carbohydrate or protein diets had little or no symptomatology. Closer examination of the composition of the diets used by these workers reveals that the high fat diet was also low in protein (approximately 9 Cal. per 100 dietary Cal. by our calculations), whereas the high carbohydrate diet had an adequate protein content (23% of the dietary Calories by our calculations). Thus the increased toxicity ob-

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TWO FIGURES

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Stimulated by the problem of the poisoning of industrial workers by trinitrotoluene (TNT) which first arose during World War I, certain investigators have studied experimentally the influence of diet on susceptibility to this compound. Voegtlin, Hooper and Johnson ('20, '21) obtained results suggesting that diet does influence the toxicity of TNT in dogs, since there were less anemia and longer survival on a bread, milk and meat diet and on meat alone than on a diet of milk and bread. Himsworth and Glynn ('42) reported that rats on a high fat diet developed severe symptoms and liver damage, while those on high carbohydrate or protein diets had little or no symptomatology. Closer examination of the composition of the diets used by these workers reveals that the high fat diet was also low in protein (approximately 9 Cal. per 100 dietary Cal. by our calculations), whereas the high carbohydrate diet had an adequate protein content (23% of the dietary Calories by our calculations). Thus the increased toxicity ob-

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tained with the high fat diets may have been caused in whole or in part by the accompanying low protein intake.

The studies reported here were designed to clarify this matter and to obtain more information on the effect of diet on resistance to TNT poisoning.

EXPERIMENTAL METHODS AND PROCEDURE

Comparison was made of the growth, appearance, blood picture and pigment excretion of TNT-treated and control

TABLE 1

Composition of diets on a caloric and on a weight basis

DIET	TYPE [1]	% BY CALORIES			100 CALORIES IN	% BY WEIGHT [2]			
		Casein	Corn oil	Cerelose		Casein	Corn oil	Cerelose	Salts
					gm				
6	LPHF	12.5	60.0	27.5	16.5	15.9	36.3	42.0	5.8
7	LPLF	12.5	6.0	81.5	25.0	10.7	2.4	83.1	3.8
8	MPHF	25.0	60.0	15.0	15.9	32.9	37.6	23.6	5.9
9	MPLF	25.0	6.0	69.0	24.6	21.7	2.5	71.9	3.9

[1] L = low; M = moderate; P = protein; F = fat.

[2] Vitamins in the following amounts were incorporated per 400 Cal. of each diet: thiamine, 2 mg; riboflavin, 2 mg; pyridoxine, 2 mg; calcium pantothenate, 5 mg; niacin, 5 mg; PGA (folvite), 0.2 mg; biotin, 0.002 mg; inositol, 2.0 mg; *p*-aminobenzoic acid, 2.0 mg; percomorph oil, three drops; menadione, 1.0 mg; α-tocopherol, 1.0 mg.

The salt mixture was the U.S.P. no. 2 formula (General Biochemicals, Inc.).

rats on each of 4 different diets varying in respect to their content of fat, protein, and carbohydrate. The composition of the diets employed is given in table 1, together with the caloric contribution of the components. Instead of the usual Atwater caloric values, we have used the more specific metabolizable caloric values per gram of 4.50 for casein, 3.75 for glucose, and 9.45 for corn oil.

TNT was incorporated in the diet in most of the experiments, since Himsworth and Glynn ('42) had used this procedure and observed that absorption was complete. The diets

were fed ad libitum and the group intake was measured three times a week. Whenever a consistent variation in the average consumption of any diet was noted, the level of TNT in that diet was altered to bring the TNT intake into line with that of the other groups, using the intake of the group on diet 6 as the baseline. The initial levels of TNT in the diets were 150 mg % for the low fat diets (7 and 9) and 300 mg % for the high fat diets (6 and 8).

For the comparative growth studies two series of animals were used; the procedure was the same in both experiments except that, during the course of series II, TNT was increased by one-third for all groups on the 31st day of TNT administration. In series I each group consisted of 5 males and 5 females; in series II 6 males were employed per group.

The TNT intakes of the groups on the different diets fluctuated with the intake of food, but the average intakes are in comparable ranges. For series I the average daily TNT intakes for the first 39 days were: diet 6 — 21.7 mg; diet 7— 21.3 mg; diet 8 — 24.1 mg; diet 9 — 24.2 mg. For series II the figures for the first 45 days were: diet 6 — 24.0 mg; diet 7 — 25.6 mg; diet 8 — 25.0 mg; diet 9 — 27.7 mg.

RESULTS

Growth

The effects on growth of the diets and the added TNT were found to be very similar in series I and II. The growth curves of the rats in series II are given in figure 1 as examples of the findings obtained.

In analyzing the effects of variations in diet composition on the toxicity of a compound, with growth as the criterion, it is necessary to take into account the effects on growth of the diets per se. Examination of the growth curves of the control rats not receiving TNT reveals: (a) the expected decrease in growth of the groups on the low protein diets (6C and 7C) below that of those on the higher protein intake (8C and 9C); (b) better growth of the male control group on the high fat-low

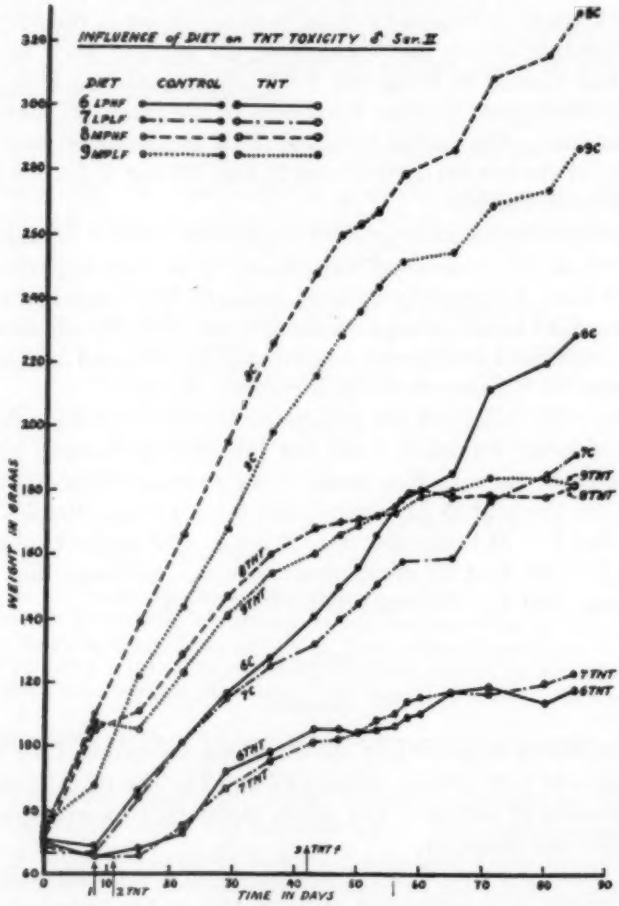

Fig. 1 The influence of TNT on the growth of rats on diets of varying composition. Average growth curves of male rats of series II. L = low, H = high, M = moderate, F = fat, P = protein, C = control group, TNT = TNT-fed group. Experimental diets started at zero time; protein increased from 7.5% to 12.5% on the 8th day (arrow 1). TNT feeding started on the 11th day (arrow 2) and increased by one-third on the 42nd day (arrow 3).

protein diet (6C) than of the low fat-low protein group (7C); the females showed no differences in the growth rates of these two groups; (c) the slightly better growth of the control group on the high fat-moderate protein diet (8C) than of the low fat-moderate protein group (9C).[2]

In examining the effects of TNT on growth the following results are apparent: (a) TNT in the amounts fed depressed growth irrespective of the type of diet ingested; at the levels of TNT employed there were no deaths in any group.

(b) Effect of protein: On the higher protein-containing diets TNT-fed rats as well as controls (diets 8 and 9) grew appreciably better than the comparable groups on the low protein diets (diets 6 and 7). This better growth might be explained on the basis of a detoxication effect of increased protein, but this seems very questionable. If there were a specific detoxication or protective effect of protein, one would expect a greater growth increment of TNT-treated rats on the higher protein intake over those on the lower protein intake than would occur between control groups on similar diets. However, it is obvious from figure 1 that the differences between the average weights of the control rats on diets 8C and 6C and between those of the animals on diets 9C and 7C at any given

[2] Attention is called to the evidence that this difference between the high and low fat diets observed with the males may not be as significant as it appears. It will be noted in figure 1 that a break in the growth curve of group 9C occurred after the animals were transferred from the pelleted stock diet to the powdery diet, and that better growth was resumed after approximately one week. Analysis of the growth rate was made by plotting the logarithm of the weight against the reciprocal of age, according to the method of Zucker et al. ('41), who found that the growth data of a normally growing rat fall on a straight line. Plotting the data of the rats on diet 8C resulted in a continuous straight line, while the data of most of the rats on 9C showed a change in slope at the point where good growth resumed. From this point on, the slope paralleled quite closely that of the rats on diet 8C. This means that after the period of faltering growth, the 9C group were capable of growing at about the same rate as the high fat group. The cause of the temporary failure in growth rate is not known. It is interesting to note that it did not occur with group 9TNT, which in this period was on the same diet since TNT administration had not yet begun. Thus, while there may have been growth differences due to the level of fat intake in the male groups on the moderate protein intake, these differences are probably less than the growth curves would indicate.

time were as large as or usually larger than the average weight differences between the TNT-treated animals on diets 8TNT and 6TNT or 9TNT and 7TNT. Thus it would appear that protein has no specific detoxicating effect. Further and more exact proof of this point requires studies on growth at other levels of protein intake, with the intake of TNT regulated according to weight and with paired feeding.

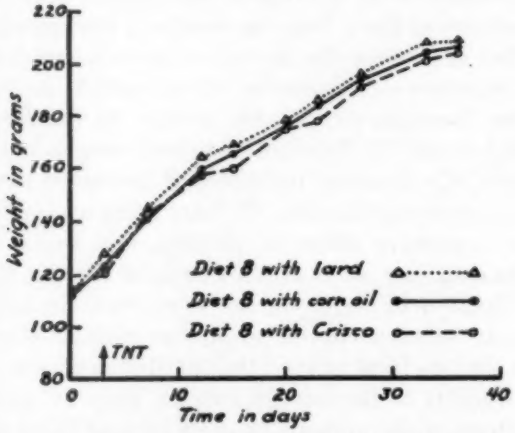

Fig. 2 The influence of different fats on the toxicity of TNT as measured by growth. Average growth curves of groups of male rats (5 per group) subsisting on diet 8 with three different sources of fat. TNT was injected daily at a level of 10 mg per 100 gm body weight after a 10-day period on the respective diets without TNT.

(c) Effect of fat: The growth curves of the series II rats (fig. 1) indicate that the groups with comparable protein levels grew at about the same rate despite the differences in fat intake when TNT was ingested. In series I (where the TNT was not increased in the course of experiment), the males on 6TNT and 7TNT had very similar growth curves throughout the experiment, while the 8TNT group tended to diverge gradually from the 9TNT group in a manner comparable to the behavior of the control groups in series II on diets 8C and 9C. The females of series I receiving TNT grew somewhat

better on the high fat diets than on the low fat diets with comparable protein intake. The differences between series I and II may indicate a somewhat greater relative toxicity on high fat diets as the intake of TNT increases.

It may be concluded from these results that TNT may be somewhat more toxic on a high fat diet than on a low fat diet *when the relative growth of the control groups is taken as a criterion.* However, it is quite clear that the level of fat in the diet is not a serious factor affecting TNT toxicity under the conditions of our experiments, in view of the fact that even when TNT was ingested at increased levels (series II), growth on the high fat diets equalled that on the low fat diets.

(d) Effect of variation in the type of fat: Because the fat used in this study (corn oil) was different from that used by Himsworth and Glynn (bacon fat), the possibility existed that the type of fat might influence TNT toxicity and so account for the differences in results obtained in the two studies. In order to check this possibility, an experiment was performed in which corn oil, hydrogenated cottonseed oil [3] and lard were compared for their effect on TNT toxicity.

Each of these fats was incorporated in a diet with the per cent composition of diet 8 (high fat-moderate protein). Five male rats were placed on each of these diets, and to insure that equal amounts of TNT were received, 10 mg of TNT dissolved in corn oil (0.3 ml) were injected intraperitoneally per 100 gm of body weight; the injections were given 6 days per week for 5 weeks. From the resulting average weight curves, which are depicted in figure 2, it is clear that the type of fat had no influence on growth.

Blood studies

At the termination of the experiments, blood samples were taken and determinations made for hemoglobin by the oxyhemoglobin method, as well as red cell counts, white cell counts and hematocrit in series I and hemoglobin alone in series II. The hemoglobin results are summarized in table 2. From

[3] Crisco.

these data it is apparent that (a) the hemoglobin of each TNT-treated group was significantly lower than that of its control group, and (b) the degree of anemia in the TNT-treated rats was statistically similar on all diets, regardless of fat, protein, or carbohydrate content. White cell counts did not appear to be influenced by TNT poisoning. Hematocrit and red blood cell counts followed the hemoglobin concentration pattern.

TABLE 2

Hemoglobin values of TNT-treated and control males of series 1 and 2

GROUP		DIET			
		6	7	8	9
1. Control	No. of rats	8	10	7	10
	Mean ± S.E. (gm %)	12.5 ± .20	12.7 ± .46	13.4 ± .37	14.2 ± .42
2. TNT	No. of rats	11	9	11	11
	Mean ± S.E. (gm %)	10.8 ± .31	10.1 ± .48	11.4 ± .34	11.0 ± .31
Comparing 1 and 2; significance of difference [1]		P = 0.001	P = 0.001	P < 0.001	P < 0.001
Comparing TNT-treated groups; significance of difference [1]		6TNT and 7TNT: P = 0.210		8TNT and 9TNT: P = 0.18	
		6TNT and 8TNT: P = 0.208		7TNT and 9TNT: P = 0.106	

[1] Calculated by Student's "t" test.

Himsworth and Glynn ('42) found that TNT caused only slight decreases in hemoglobin in the rats on their high protein and high carbohydrate diets but that a marked anemia developed on the high fat diet.

Color of urine

The excretion of a bright red urine by rats receiving TNT has been reported previously (Moore, '18; Webster, '21;

Himsworth and Glynn, '42; Channon et al., '44; Lemberg and Callaghan, '45), and was noted in this study. According to Channon et al. ('44), this pigment is trinitrobenzyl alcohol or a reduction product of it. In our experiments the urines falling on newspaper under the hanging cages of all diet groups receiving TNT were a bright red color. This observation did not conform to that of Himsworth and Glynn ('42), who noted that the rats on the "carbohydrate diet excrete a urine which at most contains traces of a pink color; rats receiving a protein diet excrete a pink urine; rats receiving a fat diet excrete a urine which looks like red ink."

TABLE 3

Effect of diet on urinary excretion of pigment following TNT administration

DIET	PER CENT TRANSMITTANCE (mean ± S.E. of the mean)
6	41.1 ± 4.9
7	46.5 ± 4.15
8	29.8 ± 3.64
9	47.4 ± 2.49

To investigate this matter further, a separate experiment was conducted in which the urine was collected from 16 rats, 4 of which were subsisting on each of the 4 types of diet (table 1) and into which were injected intraperitoneally the same quantities of TNT dissolved in corn oil. Urine was collected while the animals were in individual metabolism cages for a 24-hour period; the urines were then filtered and diluted to 100 ml and transmittance read in an Evelyn colorimeter using a 540 filter. Urine was collected twice from each rat. The first urines were obtained after a 6-day period during which the rats received 15 mg TNT daily; the second collection was made after another 7 days of daily injections of 15 mg TNT. The data for each group were averaged and the results are given in table 3. They indicate that the rats on high fat-moderate protein diet 8 excreted a more deeply pigmented urine than

those on the other three diets. Statistical analysis of variance and the "t" test indicate that the results obtained with diet 8C were significantly lower ($P < 0.001$) than the pooled data from the three other groups, which did not vary significantly among themselves. High fat per se did not cause the increased pigment, since the results with the animals on the high fat-low protein diet were the same as those obtained with the rats on low fat diets 7 and 9. Indicative of the speed of absorption of TNT from a corn oil solution in the peritoneal cavity and its metabolism in the body is the fact that within one-half to one hour following injection, the color of the urine, hitherto normal, had become bright red.

Coloration of fur

The fur of the TNT-fed rats on the high corn oil diets became stained an intense red, whereas the fur of the rats on the low fat diets had only an occasional light pink spot. Our results tend to support the view that fur coloration is of external development, presumably due to solution of the urinary pigment in dietary fat adherent to the fur, because (a) it occurred to a far greater extent in animals on a diet which caused the fur to become greasy, (b) it was much more marked on the fur around the penis, i.e., where the pigment was being excreted, (c) the pigment was not seen in the skin or other tissues but was noted in the bladder, and (d) the pigment was soluble in fat solvents as well as in urine.

This view is contrary to that of Lemberg and Callaghan ('45), who suggested that the pigment passes into the skin and hair by way of the circulation.

DISCUSSION

The question of the possible contribution of carbohydrate to the results obtained deserves mention, inasmuch as this nutrient also was varied in the diets as is shown in table 1. Because of the inverse relation of fat and carbohydrate, one can ascribe the slightly greater toxicity (as evidenced by decreased growth) of the high fat diets equally well to their low carbo-

hydrate composition as compared to the low fat (high carbohydrate) diets.

Our results are not in harmony with those of Himsworth and Glynn ('42) on a number of points. The basic discrepancy involves the fact that we found only slight differences between the influence of fat and protein on the toxicity of TNT, using many of the criteria of the English workers, whereas they found a markedly deleterious effect of fat. The fact was noted previously that their high fat diet was also low in protein; this could account for some of the effects that they ascribe to a high fat intake, such as decreased growth and a more severe anemia. However, it does not explain other differences, such as the lack of anemia they observed on the high carbohydrate and protein diets when TNT was fed, the marked differences in urine color with the different diets, the increased intake of food by the TNT-fed rats on their high fat diet, and the equal growth of the TNT-fed animals and their controls on the high protein intake.

The different types of diets employed by the British workers and ourselves may account for some of these differences, since they employed semi-synthetic diets whose components included yeast, bacon fat, wheat meal bread and casein. We have attempted to rule out the possibility of the type of fat exercising an influence by an experiment which showed no difference in growth when lard (which should be similar to bacon fat), corn oil, and hydrogenated cottonseed oil were used in the diets to which TNT was added.

Another experimental difference which possibly contributed to the discrepancies in results involves the fact that Himsworth and Glynn employed brewers' yeast in their diet at a level of 5 or 6% by weight, whereas we added synthetic "B" vitamins on an isocaloric basis. There may exist unknown factors in yeast which influence the susceptibility of the rat to TNT. Another aspect of the use of yeast by the British workers is the fact that it was incorporated in the rations at about the same level regardless of the differences in caloric densities of the three diets. On the basis of their data it ap-

pears that the levels of caloric intake of the various experimental groups receiving TNT were approximately the same. Since the high fat diet contained more than twice the calories per gram of the high carbohydrate and high protein diets, the yeast intake of the rats on the high fat diet must have been less than half that of the rats on the other two diets. Thus effects due to differences in intake of known and unknown vitamins and other nutrients may have been operating within their experiment.

Whatever the explanations for the discrepancies, the fact remains that fat per se did not influence to any marked extent the toxicity of TNT in our experiments.

It should be noted that the amounts of TNT ingested by our rats were as high as or higher than those taken by the animals in the study of Himsworth and Glynn ('42), who administered TNT at a level of 0.15 gm per kilogram of body weight.

SUMMARY

1. Observations have been made of the influence of dietary variations in protein, fat and carbohydrate on the toxicity of 2, 4, 6-trinitrotoluene (TNT).

2. TNT in the amounts fed depressed growth irrespective of the type of diet ingested. On the basis of these comparative growth studies it is concluded that a high fat intake does not appear to increase susceptibility to TNT to any marked degree.

3. No differences were noted in the effect of TNT toxicity (using growth as the criterion) when three different fats (corn oil, hydrogenated cottonseed oil, and lard) were used.

4. On the diets containing adequate protein, TNT-fed rats as well as controls grew appreciably better than on the low protein diets. The comparative growth data suggest that protein exerts no specific detoxicating effect on TNT.

5. The type of diet did not influence the hemoglobin levels of the TNT-treated animals, since all of the TNT-treated groups had an anemia of about the same severity.

6. The concentration of the red urinary pigment which was excreted following TNT injection was greater in the urine of rats on the moderate protein-high fat diet than on the other three diets, which did not differ significantly among themselves.

ACKNOWLEDGMENTS

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LITERATURE CITED

CHANNON, H. J., G. T. MILLS AND R. T. WILLIAMS 1944 The metabolism of 2:4:6 trinitrotoluene (TNT). Biochem. J., *38:* 70.

GOLDWATER, L. J., AND M. E. SHILS 1949 Some relationships of nutrition to detoxication. Am. Indust. Hyg. Assn. Quart., *10:* 17.

HIMSWORTH, H. P., AND L. E. GLYNN 1942 Experimental trinitrotoluene poisoning; the effect of diet. Clin. Sci., *4:* 421.

LEMBERG, R., AND J. P. CALLAGHAN 1945 Metabolism of aromatic nitro compounds. II. Excretion of diazotisable amines in the urine after intake of TNT and of reduction products of TNT. Austral. J. Exp. Biol., *23:* 6.

MOORE, B. 1918 The causation and prevention of tri-nitro-toluene (TNT) poisoning. Med. Res. Comm., Spec. Report, Ser. no. 11, London.

SHILS, M. E., AND L. J. GOLDWATER 1949 Influence of dietary protein and fat on the toxicities of trinitrotoluene (TNT) and 2,4 dinitrotoluene (DNT) for the rat. Fed. Proc., *8:* 397.

VOEGTLIN, C., C. W. HOOPER AND J. M. JOHNSON 1920 Trinitrotoluene poisoning. Hygienic Lab. Bull. no. 126, U. S. Govt. Print. Off., Washington, D. C.

———— 1921 Trinitrotoluene poisoning — its nature, diagnosis and prevention. J. Indust. Hyg., *3:* 229.

WEBSTER, T. A. 1921 TNT poisoning and the fate of TNT in the animal body. III. On the metabolism and excretion of 2:4:6-trinitrotoluene. Med. Res. Council Spec. Report, Ser. 58, London, pp. 49–52.

ZUCKER, T. F., L. HALL, M. YOUNG AND L. ZUCKER 1941 The growth curve of the albino rat in relation to diet. J. Nutrition, *22:* 123.

THE EFFECT ON GROWTH OF THE LEVEL OF MANGANESE IN THE DIET OF RATS, WITH SOME OBSERVATIONS ON THE MANGANESE-THIAMINE RELATIONSHIP [1]

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TWO FIGURES

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The effects of the level of manganese intake on physiological processes in white rats have been discussed in a number of papers in the past three decades. That manganese deficiency produces sterility in male rats (Orent and McCollum, '31; Waddell, '31; Skinner, Van Donk and Steenbock, '32; Becker and McCollum, '38) and in general results in less vigorous animals (Levine and Sohm, '24; Daniels and Everson, '35; Shils and McCollum, '43) is agreed by all who have investigated these subjects. But in regard to some other physiological functions, such as estrus (Orent and McCollum, '31; Skinner, Van Donk and Steenbock, '32), lactation (Orent and McCollum, '31; Skinner, Peterson and Steenbock, '31; Shils and McCollum, '43) and growth (Levine and Sohm, '24; Orent and McCollum, '31; McHargue, '26, and others), the effects of varying the manganese intake are not as well established. Often experiments in this area were designed for other purposes and observations on these functions were only incidental. Consequently, there are differences in the choice of dietary manganese levels, in the manganese compounds fed, in the duration

[1] Supported by a grant from the Office of Naval Research.

TABLE 1

Summary of reports on the effect of manganese on growth of rats

	INVESTIGATORS	MG DIETARY [1] Mn PER RAT PER DAY	Mn FED AS	AGE OR WEIGHT OF RATS WHEN DIET STARTED	DURATION OF EXPERIMENT	NO. OF RATS USED	REMARKS ON GROWTH
1	Levine and [2] Sohm ('24)	"free;" 5; 10; 25	$MnSO_4$	20–50 gm	4 weeks	?	Mn has a "marked catalytic effect on growth."
2	McHargue ('26)	low; 1.0	Mn "peptonate"	60 gm	Up to 17 weeks	8	Rats with Mn supplement gained 67% more than the controls.
3	McCarrison ('27)	Expt. I "low;" 0.56	MnO_2	40–43 days	135 days	12	MnO_2 had "a retarding influence on growth — after 32 days consumption."
		Expt. II "low;" 0.015	$MnCl_2$	Approx. 40–43 days	52 days	20	"From the outset the rate of growth of the group receiving . . . manganese chloride . . . surpassed that of the control groups."
4	Nelson, Evvard and Sewell ('29)	"low;" 0.4; 2.4	$MnSO_4$	?	56 days	?	Ave. daily gain: "low" Mn, 1.78 gm; 0.4 Mn, 2.06 gm; 2.4 Mn, 1.69 gm.
5	Orent and [2] McCollum ('31)	"Mn-free;" 0.25; 2.5	$MnCl_2$	40–50 gm	"to maturity"	?	"The growth curves of rats fed the manganese-free diet did not differ from those of well-nourished rats."
6	Skinner ('32)[3]	Expt. I "Moderate;" 10	$MnSO_4$	Mothers on diet from breeding time	to weaning	193	Young of Mn-supplemented mothers ave. 40.4 gm at 21 days. Young of control mothers ave. 37.0 gm at 21 days.
		Expt. II "Moderate;" 2–10 [3]	$MnSO_4$	23–26 days	12 weeks	40	"Growth and utilization of food were practically unaltered."
7	Becker and [2] McCollum ('38)	5; 10; 25; 50; 100	$MnCl_2$	40–50 days	240 days	?	"On all diets except where $MnCl_2 \cdot 4H_2O$ was fed at 3.6% (100 mg) the animals grew well and produced healthy young."
8	Boyer, Shaw and Phillips ('42)	0.003; 1.0	$MnCl_2$(?)	— [4]	46–47 weeks	14	Growth curves started at about 25 gm wt. Mn-supplemented rats consumed about 20% more food. "The growth obtained per 100 ml of milk consumed . . . was 2.32 gm for the low manganese group . . . and 2.84 gm" for the Mn-supplemented group.
9	Shils and McCollum ('43)	0.003; 10	$MnCl_2$	21 days [5]	33 weeks	36	"Manganese is essential for optimum growth."
10	Wachtel, Elvehjem and Hart ('43)	0.004–0.005; 0.05	$MnSO_4$	14 days	4–6 weeks	78	"In a mild deficiency growth is normal." "In a more severe manganese-deficiency the animals show a definitely impaired growth."

[1] Where author does not give Mn of diet in milligrams per rat per day, amount was calculated on the basis of 10 to 12 gm solid food intake per day per adult and 3 to 6 gm at 20 to 40 days of age.
[2] No detailed data or growth curves presented.
[3] Two milligrams Mn first 7 weeks, 10 mg Mn following 5 weeks.
[4] Mothers "on the low manganese ration during the last few days of gestation and during the suckling period."
[5] Mothers had been on Mn-deficient diet for one week prior to birth of young. At weaning, half the young were placed on a 10-mg Mn intake; the other half were kept on controls.

of manganese deficiency and, perhaps most important, in the age of the experimental animals when the dietary deficiency was started. For these reasons there is in some cases no sound basis for comparison between two papers, both of which present conclusions with regard to the influence of the level of manganese intake on the same physiological functions.

The data in the literature reporting the effects of dietary manganese on growth, the primary interest of this paper, are summarized in table 1. The table is probably complete, omitting only certain papers in which the immediate interest was the study of other functions and the data on growth so complicated by other factors as to make comparative interpretation difficult. It will be seen that in only a few cases were more than two levels of dietary manganese used, and that few analyses for manganese were made in the "low" manganese diets. In no case was an effort made to establish the optimum intake of manganese for growth.

In the work reported here our purpose has been to measure the optimum intake of manganese for growth by the use of 7 different levels of manganese intake. Perla and his associates (Perla, '39; Perla and Sandberg, '39; Sandberg, Perla and Holly, '39; Perla, Sandberg and Holly, '39) have suggested that there is a reciprocal relationship between manganese and thiamine in physiological processes, and have offered data to support this view based on reciprocal detoxification experiments and on the rate of manganese depletion in rats on different levels of thiamine intake. For this reason we have added thiamine to the diet at two different manganese levels in an attempt to show whether any antagonism between dietary manganese and thiamine is evident with respect to the growth function.

EXPERIMENTAL

Powdered whole milk was used in the basal diet because of its very low manganese content. Further, a barrel of powdered whole milk assures an unvarying supply, sufficient for a whole experiment. The preparation and supplementation of the basal diet are given in table 2.

TABLE 2

Composition of diets

BASAL DIET		SUPPLEMENTS TO DIETS	
Component	Amount	Diet no.	Supplement
			per 100 ml milk
Powdered whole milk [1]	121.5 gm	I	none [5]
Iron, as $FeSO_4$	50 mg	II	0.1 mg Mn
Copper, as $CuSO_4$	1 mg	III	1.0 mg Mn
Corn oil [2]	10 ml	IV	5.0 mg Mn
Vitamin D_2 [3] (dissolved in the corn oil just before using)	150 units	V	10.0 mg Mn
		VI	1.0 mg Mn + 0.1 mg thiamine
Thiamine [4]	1 mg	VII	10.0 mg Mn + 0.1 mg thiamine
Niacin [4]	1 mg	VIII	20.0 mg Mn
p-Aminobenzoic acid	180 mg	IX	40.0 mg Mn
Folic acid [4]	50 μg	X	Stock diet [6]

[1] Spray-process dried.
[2] Mazola.
[3] Vitamin D_2, obtained as Drisdol, Winthrop Chemical Co., New York, N. Y.
[4] Thiamine, niacin (as nicotinic acid amide) and folic acid were supplied by the Lederle Laboratories, Pearl River, New York, through the courtesy of Dr. Guy Clark.
[5] Contains about 0.03 mg Mn per 100 ml.
[6] Purina Fox checkers, water ad libitum and lettuce three times per week.

The dried milk and oil were mixed in a Waring Blendor with approximately 500 ml of distilled water to which the minerals had been added previously. The other vitamins were added to the contents of the Waring Blendor just before mixing. After blending, the mixture was made up to 1,000 ml with distilled water. This diet was made fresh each day. No settling or breaking of the homogeneous emulsion occurred. When manganese supplements were used, they were added to the water with the other minerals before blending. Thiamine was added to the basal diet at the level of 1 mg per 1,000 ml because preliminary experiments showed that reproduction and rearing of the young were unsatisfactory at lower levels. Additional thiamine supplements (diets VI and VII, table 2) were added to the thiamine of the basal diet. In all, 10 different diets were used, as listed in table 2.

Diets I to IX were fed from inverted bottles equipped with drinking tubes to insure against contamination and loss. No additional water was offered. Adult rats consumed about 100 ml of this fortified, reconstituted milk per day.

The rats used were from our colony, which was originally of Wistar stock and has been inbred in this institution for many years. Young adults, 8 males and 8 females for each of the 9 milk diets, were placed on the respective diets 15 days before the males were caged with the females (one male and one female per cage). Before the birth of the young, the females were removed to individual cages containing rayon waste as bedding. The litters were reduced to 6 if they exceeded that number and were first weighed on either the second or third day of life. Three more weighings were made before weaning. The plan was to use three of each litter for the growth experiments; that is, 24 in each group. The smaller numbers set forth in figure 1 reflect the efficiency of reproduction on the different diets.

RESULTS AND DISCUSSION

The results of these experiments are shown in figure 1. The solid lines represent the average growth curves on the various diets. The broken lines that transect these represent the aver-

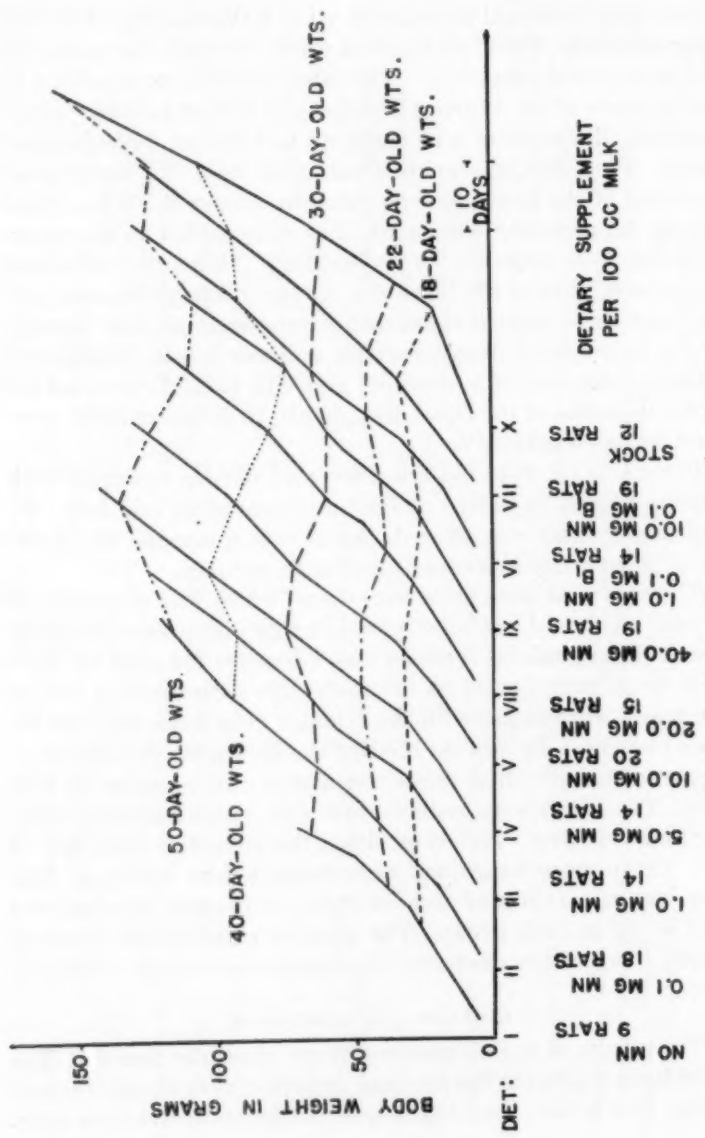

Fig. 1 Comparison of average growth rate curves.

age weights of the groups at the ages indicated. Until the 18th day the manganese intake of the young was entirely by way of the mother's milk. At this age the young were first seen to reach for the feeding tubes of the diet bottles. At 30 days of age the young rats were weaned and from that time on were dependent entirely on the prepared milk diet. No change was made in the ration offered. In this experiment, then, there were three dietary periods after the birth of the young: (1) birth to 18 days, the period during which food was taken only by "mother passage." So far as we know, no analyses of rat milk for manganese have been made; however, judging from the low manganese content of cow's milk, this must be a very inefficient means of making dietary manganese available to the young. (2) From 18 to 30 days, a period of transition, when the young were still partly dependent on the mother's milk but had increasing access to her ration. (3) The period of complete dependence on the prepared diet. In the last period the manganese intake was unquestionably greatest. It will be seen from figure 1 that, at 18 days of age, there was little difference in the average weights of the groups, at least up to the 10-mg level of daily manganese intake of the mother. But as soon as the young had access to the mother's diet an advantage appeared for the 5.0-mg dietary level. The advantage of this diet (diet IV) became more evident as the experiment progressed. The adult rats consumed about 100 ml per day. The dietary manganese values as listed in figure 1 are, therefore, the average daily intakes of the mother rats. At the weaning age of 30 days the young consumed an average of 35 to 45 ml per rat per day. The consumption continued to increase to an average of 70 to 80 ml per rat at 50 days of age. Thus diet IV, which gave the best growth response, provided 2.0 mg Mn per day for the average rat at 30 days of age, increasing to 3.75 mg per day for the average rat at 50 days of age.

In figure 1 the difference in slope between adjacent curves is small, though the trend is evident. However, if the curves of diets IV and IX are compared, as is shown in figure 2, and

subjected to statistical analysis using the "t" test for unpaired variates (Freeman, '47), the difference is significant ($P < 0.01$). It will be seen that after the 18th day no weighing for diet IX was as high as the average for diet IV and that only 4 weighings of diet IV approximated the average of diet

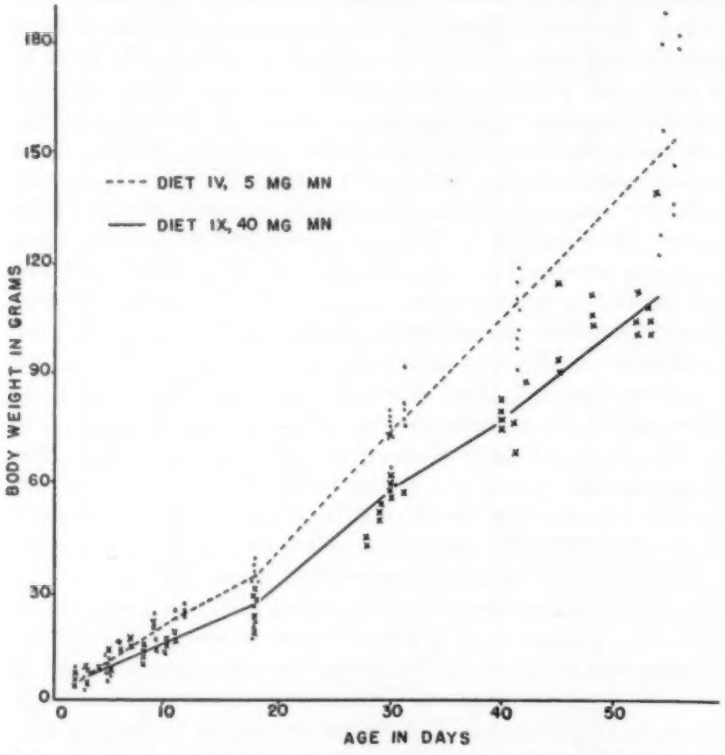

Fig. 2 Comparison of average growth rates for young on diets IV and IX.

IX. Diets VI and VII differ from diets III and V, respectively, only in that they have additional thiamine added at the rate of 0.1 mg per day per 100 ml of diet. This amount of thiamine, bringing the total to double that of the basal diet, made no appreciable difference in the growth rate. At these levels of

intake there is no evidence of antagonism between manganese and thiamine with respect to growth.

Diet X, a commercial animal food [2] plus lettuce three times a week, was included as a check on the general quality of the diets. It is obvious from the growth response on these diets that the milk diets are inferior to diet X after the weaning period; before weaning, however, they are as good and perhaps better.

SUMMARY

Seven different levels of manganese intake have been used in an effort to establish the optimum dietary manganese level for growth in white rats.

A wide range of manganese intake (0.1 to 20.0 mg per mother rat per day) is permissible during the lactation period. This is probably a reflection of the inefficient passage of dietary manganese into the milk, as well as of inefficient absorption from the intestinal tract of the mother. The young, therefore, have a lower level and narrower range of manganese intake than are apparently provided by the diet of the mother.

After weaning, the optimum manganese intake for growth is 2 mg per rat per day at 30 days of age and about 3.75 mg per day at 50 days of age.

At the levels fed in this experiment there is no evidence of antagonism between dietary manganese and thiamine with respect to the growth function.

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LITERATURE CITED

BECKER, J. E., AND E. V. MCCOLLUM 1938 Toxicity of $MnCl_2$, $4H_2O$ when fed to rats. Proc. Soc. Exp. Biol. Med., *38:* 740.

BOYER, P. D., J. H. SHAW AND P. H. PHILLIPS 1942 Studies of manganese deficiency in the rat. J. Biol. Chem., *143:* 417.

[2] Purina checkers.

DANIELS, A. L., AND G. L. EVERSON 1935 The relation of manganese to congenital debility. J. Nutrition, *9:* 191.

FREEMAN, H. A. 1947 Industrial Statistics. John Wiley and Sons, Inc., New York, p. 48.

LEVINE, V. E., AND H. A. SOHM 1924 The effect of manganese on growth. J. Biol. Chem., *59:* xlviii.

MCCARRISON, R. 1927 The effect of manganese on growth. Indian J. Med. Res., *14:* 641.

MCHARGUE, J. S. 1926 Further evidence that small quantities of copper, manganese and zinc are factors in the metabolism of animals. Am. J. Physiol., *77:* 245.

NELSON, V. E., J. M. EVVARD AND W. E. SEWELL 1929 The effect of manganese on the growth of rats. Proc. Iowa Acad. Sci., *36:* 267.

ORENT, E. R., AND E. V. MCCOLLUM 1931 Effects of deprivation of manganese in the rat. J. Biol. Chem., *92:* 651.

PERLA, D. 1939 The prevention of toxic manifestations of an excess of vitamin B_1 by supplements of manganese to the diet. Science, *89:* 132.

PERLA, D., AND M. SANDBERG 1939 Metabolic interdependence of vitamin B_1 and manganese. Reciprocal neutralization of the toxic effects. Proc. Soc. Exp. Biol. Med., *41:* 522.

PERLA, D., M. SANDBERG AND O. M. HOLLY 1939 Interdependence of vitamin B_1 and manganese. III. Manganese, copper and iron metabolism in normal rats. Ibid., *42:* 371.

SANDBERG, M., D. PERLA AND O. M. HOLLY 1939 Interdependence of vitamin B_1 and manganese. II. Manganese, copper, iron metabolism in B_1 deficient rats. Ibid., *42:* 368.

SHILS, M. E., AND E. V. MCCOLLUM 1943 Further studies on the symptoms of manganese deficiency in the rat and mouse. J. Nutrition, *26:* 1.

SKINNER, J. T. 1932 The effect of a high intake of manganese on the growth of rats. Ibid., *5:* 451.

SKINNER, J. T., W. H. PETERSON AND H. STEENBOCK 1931 The manganese metabolism of the rat. J. Biol. Chem., *90:* 65.

SKINNER, J. T., E. VAN DONK AND H. STEENBOCK 1932 Manganese as a factor in reproduction. Am. J. Physiol., *101:* 591.

WACHTEL, L. W., C. A. ELVEHJEM AND E. B. HART 1943 Studies on the physiology of manganese in the rat. Ibid., *140:* 72.

WADDELL, J. 1931 Male sterility on milk diets. J. Nutrition, *4:* 67.

THE RIBOFLAVIN REQUIREMENT OF THE GROWING PIG AT TWO ENVIRONMENTAL TEMPERATURES

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THREE FIGURES

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The determination of the requirement of an animal for any specific nutrient presents technical difficulties, particularly in deciding on a proper criterion of nutritional adequacy with reference to the nutrient in question. The biological variation in dietary requirements, due to variation in requirement from animal to animal, is also a part of this difficult problem. The effect of this variation on an average requirement applicable to the population of animals from which the experimental animals were drawn can be minimized by selecting a sufficiently large sample of the population, and, at times, by standardizing them through subjecting them to an appropriate pre-treatment. The residual effect of this biological variation on the mean requirement can be assessed by the calculation of a standard error of the mean. Thus, Steggerda and Mitchell ('46), summarizing the results of a series of experiments on the determination of the dietary calcium requirement of adult human subjects (totaling 43 in number) on comparable diets, deduced an average requirement of 365 mg per square meter of body surface per day, for which a standard error of $\pm$ 12 mg may be computed. The coefficient of variation of individual determinations was 21.8%, a composite variation that would include technical

errors and differences in individuality. But the conditions for such a complete statement of a dietary requirement would rarely be realized in a single experiment.

The distressing variation exhibited by different rats in assays for vitamin D has led investigators to study all possible factors, with reference to the selection of test animals and to the environment, that might be brought under better control. From a statistical study of their experience with this assay, Tourtellotte and Bacon ('35) suggested that room temperature may be a factor, since their results indicated, albeit not very strongly, that rats are more sensitive to rickets at room temperatures of 80°F. or above. Two years later Guerrant, Dutcher and Crowthers ('37) subjected this suggestion to systematic study. Although they concluded "that environmental temperature is not an important consideration in either the production or the cure of rickets in the rat," if the implications of the differences in food intakes of the rats in the different environments are fully considered, the experiment may be interpreted as a confirmation of the suggestion that vitamin D requirements are increased in a hot environment.

Since these experiments were published, the effect of environmental temperature on vitamin requirements has been subjected to considerable study, mainly due to the stimulus provided by the publications of Mills. Rats were used as subjects in all of Mills' work. The results are confusing in their significance, to some extent because of the experimental methods used or of debatable interpretations. The impression conveyed by the published reports is that environmental temperature is probably a factor which should be considered in determining the requirements of animals for some of the vitamins.

The effect of environmental stress on nutrient requirements seems worthy of serious study. Using an experimental chamber in which temperature can be controlled within 2°F., and relative humidity within 5%, we have started an investigation in this field in which growing pigs have served as sub-

jects. The first nutrient to be studied was riboflavin. The only previous attempt to determine the effect of temperature on the requirement for riboflavin of which the authors are aware was reported by Mills ('43), using growing rats on different levels of riboflavin in two different experimental rooms, one a cool room maintained at 65°F., and one a hot room maintained at 91°F. and 60% relative humidity. From the fact that the minimum level of riboflavin inducing the most rapid average gain in groups of 4 rats seemed to be the same in the two environments, i.e., 2 mg per kilogram of diet, Mills concludes that the temperature difference exerted no effect on riboflavin requirements. The rats were fed ad libitum and the average data were not submitted to statistical analysis. Hence, a critical appreciation of them is difficult. The average food economy of gains in body weight is not obviously inconsistent with the conclusion that in the hot room the riboflavin requirement was nearer 1 mg per kilogram of diet than 2 mg.

The experiments to be reported below afford definite proof that in the growing pig the greater requirement of riboflavin is associated with the cooler environment.

METHODS

The general plan of the two experiments to be reported was to place the experimental pigs on a semi-synthetic basal ration designed to contain very little riboflavin. The ration consisted of: glucose 28.75, starch 28.75, rolled oats 10, vitamin-free casein [1] 20, riboflavin-free vitaminized starch 5, sodium chloride 1, limestone 1, bone meal 1, cod liver oil 1.5, salt mixture (calcium-free) no. 794, 1, and wood flock 2%. The vitaminized starch contained, per kilogram, 50 mg of thiamine, 50 mg of pyridoxine, 400 mg of calcium pantothenate, 200 mg of nicotinamide, 1 gm of para-aminobenzoic acid, 2 gm of inositol, and 20 gm choline.[2] The mineral mixture contained salts of potassium, magnesium, manganese,

[1] Labco.

[2] As Merck's dry mix.

iron, aluminum, copper and cobalt, phosphorus as dipotassium phosphate, and traces of potassium iodide and sodium fluoride. By analysis of samples of this ration, taken periodically throughout the experiments, it was found to contain from 0.2 to 0.4 μg of riboflavin per gram of ration (p.p.m.). The samples were prepared for the microbiological determination of riboflavin according to the directions of Strong and Carpenter ('42), and the determinations were carried out by the method of Snell and Strong ('39).

In both experiments the pigs were fed this depletion diet for three weeks, at which time they were given graded doses of the vitamin, as described below, except for one pig in each experiment, which was continued on the basal diet alone.

Each experiment included 8 pigs, since the chamber would not accommodate more. They were fed in individual metabolism crates, and the food intakes of all 8 pigs were equalized to the intake of the pig consuming the least, until the pigs on the lower levels of riboflavin showed deficiency symptoms: anorexia, vomiting, rough hair coat, inability to stand normally. Diarrhea was not a prominent symptom of riboflavin deficiency, and at no time were cataracts or corneal changes observed on examination with the ophthalmoscope. When signs of deficiency appeared, the food consumption of the unaffected pigs was not restricted to that of the deficient pigs.

The pigs were weighed weekly before a morning feeding, and blood samples were taken at the beginning and end of the experiment for cell counts and hemoglobin determinations.

Experiment 1

In this experiment, 8 Duroc-Jersey pigs, weighing initially from 55 to 61 lb. (25 to 29 kg), were maintained for approximately 10 weeks at an environmental temperature of 85°F. and a relative humidity of 50%. They were all castrated males and were from two to two and one-half months old at the start of the experiment, which lasted from May 17

to July 23, 1948. After the three-week depletion period on the basal ration only, they were given riboflavin supplements at each of the two daily feedings to raise the concentration of the vitamin in the air-dry feed to the following levels, in milligrams per kilogram, or p.p.m.: 0.2 (no supplement), 0.7, 1.2, 1.7, 2.2, 2.7, 3.7, and 5.2. The ratio of water consumed to food consumed was maintained at 4:1 in both tests; i.e., the amount of food allotted each pig determined the water intake.

After 5 weeks of feeding at these levels of riboflavin, the pigs were pair-fed for the final two weeks in order to subject to a second test the conclusion as to the required level of riboflavin based on the results of the first 5 weeks. During this two-week period, the levels of riboflavin compared with respect to growth promotion were 0.2 vs. 3.7, 0.7 vs. 5.2, 1.2 vs. 1.7, and 2.2 vs. 2.7 p.p.m.

Experiment 2

Eight Hampshire pigs, from the same litter, served as the subjects of this experiment, the sexes being equally divided. These pigs were about 9 weeks old when received at the Nutrition Laboratory. During this experiment, the chamber was kept at 42°F. and 70% relative humidity. After a three-week depletion period, the pigs were placed individually on diets containing the following levels of riboflavin: 0.38, 0.83, 1.34, 1.83, 2.33, 2.83, 3.33, and 4.34 p.p.m. of ration (milligrams per kilogram). For the next 40 days they performed poorly in food consumption and in growth, with no clear distinction between pigs on the lower levels of riboflavin and those on the higher levels. Since fecal examination revealed the presence of intestinal worms in all of the animals, they were given 1.5 gm of sodium fluoride per pig at this time. Immediately their appetites improved and they gained in weight at a more rapid rate. The experimental period for which reports will be made, following the deworming operation, extended for 7 weeks, from January 8 to March 5, 1949. The initial weights of these pigs, due to

their poor performance up to this period, were smaller than those of the pigs in experiment 1, i.e., 32 to 38 lb. (14 to 17 kg).

Besides the main experiments described above, brief reference will be made to some preliminary and auxiliary experiments on larger pigs, designed to determine the effect of cold and of dietary supplements of protein, carbohydrate and fat on the urinary output of riboflavin. In these tests, the florisil adsorption-elution procedure, as described by Johnson ('48), was used in determining the riboflavin content of urine.

EXPERIMENTAL RESULTS

The first indication that environmental temperature may be a factor in determining the requirement of growing pigs for riboflavin was secured in some preliminary experiments on 11 pigs weighing from 120 to 260 lb. In these pigs the output of riboflavin on the same ration was observed at 85°F. and 38° to 40°F. In all pigs the output was greater in the heat than in the cold, suggesting, though not proving, that the requirement is smaller in the heat than in the cold, on the assumption that the urinary output is a spill-over of intake consumed above current needs. The change from a cold to a hot environment, or the reverse, was always associated with a change in the rectal temperature of the pig, averaging 1.24°F. for contiguous 4-week periods, the higher temperature being associated with the hot environment.

Experiment 1

The most important results of experiment 1 are assembled in figure 1. The results for each of the 8 pigs at the end of 5 weeks of feeding are spaced from left to right in accordance with the concentration of riboflavin in the 8 experimental diets. The two pigs receiving the lowest concentrations of the vitamin showed definite external deficiency symptoms in appearance (hair coat) and behavior (ability to stand and move around). The pig on the lowest level of riboflavin

exhibited persistent vomiting. The occurrence of diarrhea was unrelated to vitamin intake and when particularly persistent in pigs 2, 5, and 7, indicating intestinal infectior, it was treated with sulfasuxidine. The slightly lower intake of food of pigs 1 and 2 on the lowest vitamin levels was associated with a slower gain in body weight, as compared with all of

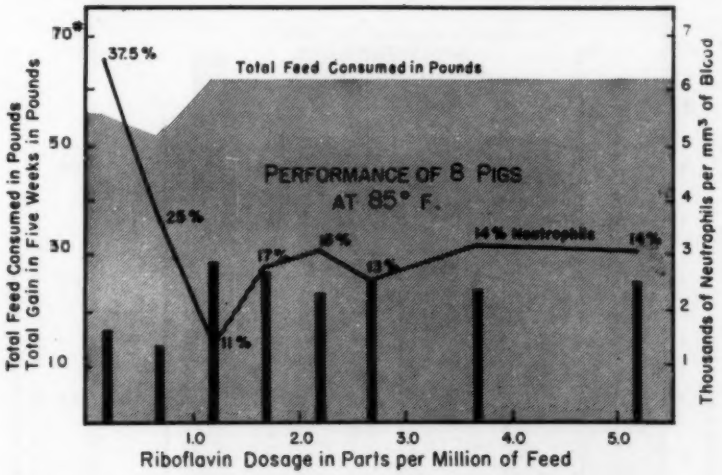

Fig. 1 The results of the first 5 weeks of experiment 1 for the 8 pigs in the group are given above the respective points on the horizontal dosage scale: total gains in body weight and total feed consumed on the left-hand scale, the final concentration of neutrophils in the blood on the right-hand scale; the final percentages of neutrophils in the total leukocyte counts are given in the body of the chart. The total food consumption is represented by the upper border of the shaded area in the chart.

the other pigs on higher levels of riboflavin. The average daily gains of the pigs in the first 5 weeks of this experiment were, in the order of increasing vitamin dosage, as follows: 0.46, 0.37, 0.81, 0.77, 0.64, 0.76, 0.70, and 0.71 lb. In the same order, the food required per pound of gain was 3.57, 4.07, 2.20, 2.39, 2.74, 2.33, 2.51 and 2.47 lb. The average rectal temperature of the pigs was 104.1°F.

Referring again to figure 1, it will be noted that the first two pigs, as compared with the remaining 6, showed greater concentrations of neutrophils in the blood and greater percentages of neutrophils in the total leukocytes. If this may be considered an abnormal phenomenon because of its association with impaired appetite, unthrifty appearance and muscular weakness, the blood data confirm the conclusion that

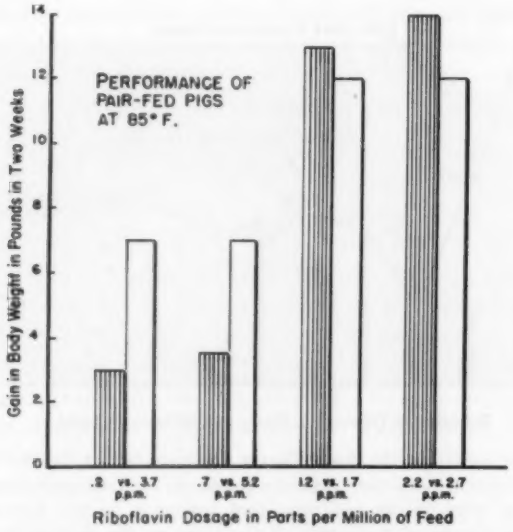

Fig. 2 The gains in body weight of the pigs in experiment 1 during the last two weeks, when the pigs were paired as indicated and food consumption equalized within pairs.

the requirement of riboflavin in this hot environment lies between 0.7 and 1.2 p.p.m. of ration.

During the last two weeks of the experiment the pigs, with no change in riboflavin dosage, were pair-fed to test further the conclusion supported by the observations of the first 5 weeks. The pairing was carried out in such a way that the lower levels of vitamin intake were compared with the higher,

with food intakes the same within pairs. The results are shown graphically in figure 2. They confirm the conclusion that the levels of 0.2 and 0.7 p.p.m. of riboflavin were inadequate for maximum growth. No evidence was secured that the 1.2 and 2.2 p.p.m. levels were inadequate.

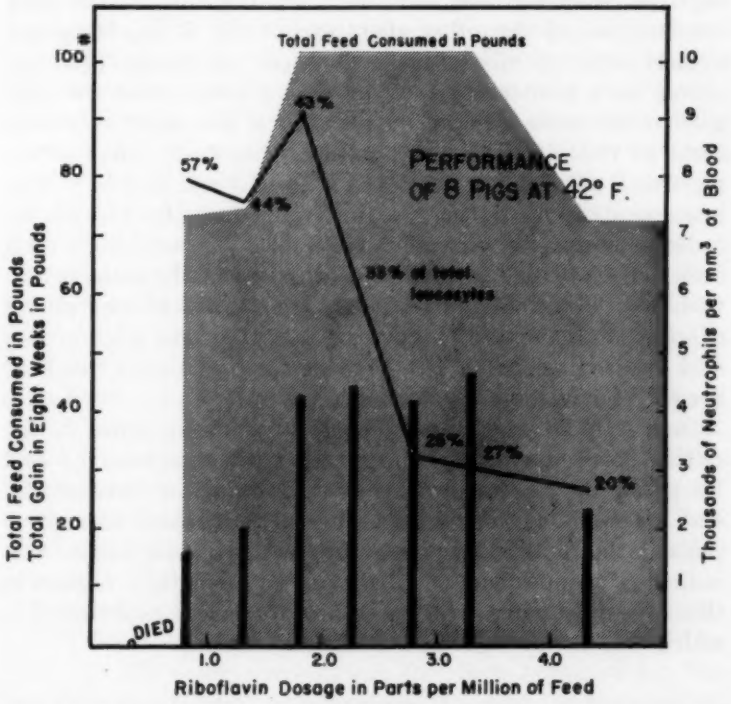

Fig. 3 Results of the 8 weeks of experiment 2 for the 8 pigs in the group are given above the points on the horizontal scale corresponding to the riboflavin dosages given: total gain in body weight and total food consumed on the scale at the left, the final concentration of neutrophils in the blood on the scale at the right; the final percentage of neutrophils in the total leukocyte counts are given at the corresponding points on the neutrophil-count curve. The total food consumption is represented by the upper border of the shaded area in the chart.

Experiment 2

The most significant results of this experiment are presented in figure 3, which has been constructed in the same manner as figure 1. In this group, the pig on the lowest level of riboflavin died on the 6th day of the experimental period. The two pigs on the next highest levels showed the usual signs of deficiency and thereafter did not restrict the food consumption of the other pigs, except pig 8, receiving the highest level of riboflavin (4.34 p.p.m. of food). This pig served as a positive control for pigs 2 and 3, and although held to the same food consumption level she never exhibited signs of vitamin deficiency, while gaining faster than either pig 2 or 3 (23.0 lb., as compared with 15.5 and 20.0 lb.). The average daily gains of the pigs, omitting pig 1, were in order of increasing vitamin dosage, 0.28, 0.36, 0.77, 0.79, 0.72, 0.84, and 0.41 lb. The food cost of gains, in the same order, was 4.68, 3.72, 2.60, 2.51, 2.76, 2.37, and 3.17 lb. per pound of gain. The average rectal temperature of the pigs in this cold environment was 102.8°F., as compared with 104.1°F. for the pigs in the hot environment.

On the basis of the assemblage of data in figure 3, the critical level of riboflavin in the ration lies between 1.3 and 1.8 p.p.m. as judged by body weight gains, or between 2.3 and 2.8 p.p.m. as judged either by the percentage of neutrophils in the total leukocytes or by the neutrophil count. The indicated requirement of riboflavin was definitely higher in this experiment in a cold environment than in experiment 1, which was carried out in a hot environment.

DISCUSSION

The plan of feeding employed in these experiments was in part dictated by the amount of air-conditioned space available. However, it proved to have its peculiar advantages, since in effect it divided the experimental pigs into two lots, one receiving inadequate levels of riboflavin and the other adequate levels, the division being determined by the require-

ments of the pigs for the vitamin. If the division of the pigs into two lots had been predetermined in a more or less arbitrary fashion, quite probably the most significant division level would not have been chosen.

The most sensitive index of riboflavin deficiency observed in these experiments was the concentration of neutrophilic granulocytes in the blood and their percentage of the total leukocyte count. These leukocytes are produced in the bone marrow and are known as microphages because their phagocytic activities are directed toward bacteria and small particles (Wintrobe, '47). Their metabolism, as measured by consumption of oxygen and sugar, is at least twice as great as that of the lymphocytes. An increase in neutrophils in riboflavin deficiency has been noted also by Shukers and Day ('43) in the growing rat, prior to the appearance of changes in the lens and cornea of the eye, but was attributed by these authors to partial inanition since it was also evident in isocaloric control rats receiving adequate riboflavin. On the other hand, Ershoff and Adams ('46) observed no effect of reduced caloric intake on the granulocyte count of mature female rats when the diet was fortified with B vitamins. On a diet not so fortified, partial inanition caused a reduction in granulocyte count, which could be obviated by a supplement of folic acid. Many types of stress, as these authors point out, induce a granulocytopenia rather than a granulocytosis.

Pyridoxine deficiency has been observed by Weir, Heinle and Welch ('49) to induce granulocytosis and lymphopenia in mice. The same phenomenon has been reported for the dog (Morgan, Groody and Axelrod, '46) and for the monkey (McCall et al., '46), but it was not noted in pyridoxine-deficient pigs (Wintrobe et al., '43). McCall et al. ('46) also noted a reversal of the neutrophil-lymphocyte ratio in the blood of the monkey suffering from a deficiency of the anti-anemia factor.

In the experiments reported in this paper, the granulocytosis preceded any changes in the eye which could be detected

upon examination with the ophthalmoscope or in the skin, such as those observed by Patek, Post and Victor ('41) and Wintrobe et al. ('44). It was not due to partial inanition, since it did not appear in pig 8 of experiment 2, whose intake of food was restricted to that of pigs 2 and 3 (see fig. 3).

TABLE 1

The riboflavin requirements of animals of different species expressed as concentrations in the air-dry diet

SPECIES	ESTIMATED RIBOFLAVIN REQUIREMENT IN MG PER KG OF DIET (P.P.M.)	AUTHORITY
Mouse, growing	4 [1]	Fenton and Cowgill ('47)
Rat, growing	2 [2]	Mills ('43)
Duckling	between 3 and 4	Hegsted ('48)
Chick	3.0 to 3.5	Bolton ('47)
Chick	2.7 to 3.2	Bird et al. ('46)
Poult	3.2 to 3.8	Bird et al. ('46)
Dog, growing	1.5 to 2.5	Potter, Axelrod and Elvehjem ('42)
Fox, growing	1.2 to 4	Schaefer, Whitehair and Elvehjem ('47)
Pig, growing	3.1	Krider, Terrill and Van Poucke ('49)
Pig, growing	1.2 to 2.3	This experiment, depending on environmental temperature
Human, infant	between 2.5 and 3.8 [3]	Snyderman et al. ('49)
Human, child	2.2 [4]	Oldham et al. ('44)
Human, adult	1.6 to 2.3 [4]	Williams et al. ('43)
Human, adult	1.3 to 2.3 [4]	Horwitt et al. ('49)

[1] One strain of mice, C_{57}, may have required somewhat less than 4 p.p.m. of riboflavin, and the other strain, A, required somewhat more, but the results on the latter strain were not reproducible.

[2] Possibly somewhat less in a warm environment.

[3] Calculated from data given in article.

[4] Estimating the diet to contain 4.5 cal. per gram.

Neither the hemoglobin concentration of the blood nor the erythrocyte count was affected by the riboflavin deficiency imposed in these experiments.

The expression of riboflavin requirements in terms of concentration in the ration (dry or air-dried) rather than per

unit of weight, as is so commonly done, seems rational in view of the known functions of the vitamin in the body. By reason of its participation in enzyme systems involved in carbohydrate, protein and purine metabolism, riboflavin functions in the body to facilitate the liberation of food energy and the assimilation of food nutrients. Hence, the requirement of riboflavin would be in proportion to the amount of nutrients oxidized in the body or otherwise utilized, and not to body weight or to rate of growth except as these determine food consumption. The relative proportions of the main organic nutrients in the diet may also be determinants (Mannering, Lipton and Elvehjem, '41; Riesen, Schweigert and Elvehjem, '46), directly or through differential effects of the organic nutrients on intestinal synthesis (Czaczkes and Guggenheim, '46), or both.

Other evidence possessing the same significance may be found in the similarity in the riboflavin requirements of animal species of different size when the requirement has been expressed as a dietary concentration or when it can be so computed from the reported data. A compilation of such evidence, collected from the recent literature, is presented in table 1. While differences in the reported requirements of riboflavin by different species of animals are evident from this compilation, they are not related to body size; in all likelihood they are traceable to differences in the criteria of adequacy employed, to differences in the length of feeding periods, to differences in basal diets, and to differences in environmental temperature.

An important corollary of the conclusion that the requirement for riboflavin is in proportion to the amount of food to be metabolized is that requirement for the vitamin per unit of diet is independent of the rate of growth secured under the conditions of experimental feeding.

The riboflavin requirements of this experiment, somewhat less than 1.2 p.p.m. in a warm environment (85°F.) and approximately 2.3 p.p.m. in a cold environment (42°F.), are both less than the value of 3.1 p.p.m. (1.4 mg per pound)

deduced by Krider and associates ('49) from experiments carried out under uncontrolled environmental conditions and with uncontrolled food consumption. The two experiments on which this value is based, however, suffer from two unfortunate circumstances. In the first experiment, the average daily gains do not increase evenly from the group on the basal diet to that receiving 1.5 mg of the vitamin per pound. The results indicate merely that the level of 0.5 mg of riboflavin per pound of feed was inadequate for maximum gains. In the second experiment, in which the basal diet was assumed to have provided over 1 mg of riboflavin per pound, its precise content of the vitamin was not determined, the value used in the computations being the average of two discordant results secured in different laboratories. The recommended riboflavin allowance of the Committee on Animal Nutrition of the National Research Council for growing and fattening pigs (Hughes et al., '44) is 0.76 mg per pound of feed, or 1.7 mg per kilogram. None of the practical rations recommended by this Committee for pig feeding contained as much as 1.4 mg of riboflavin per pound.

Hughes ('40) has assessed the requirements of the growing pig as being between 1 and 3 mg per 100 lb. body weight on the basis of two experiments, each involving 25 pigs on synthetic diets. The rate of growth on uncontrolled food consumption was used as the measure of nutritive adequacy. The results of the experiments reported in this paper, expressed on a body weight basis, are 1.4 mg and 4.2 mg per 100 lb. body weight for the warm and the cold environments, respectively.

Speculation as to why riboflavin requirements are higher in a cold than in a warm environment, in spite of a lower body temperature, may be pursued along three lines. First, they may be the result of a greater loss of riboflavin in the urine in the cold environment. The ratio of water to food consumed in both environments was the same, i.e., 4 to 1. Presumably the water vaporized from the skin was less in the cold environment, leaving more water to be excreted

by the kidneys. The experiments of Feder, Lewis and Alden ('44) imply a high correlation between urine volume and riboflavin output through the kidneys. However, so far as the present authors are aware, this correlation has not been observed by others.

Secondly, the metabolism of the pigs in the cold environment probably was elevated above that of the pigs in the warm environment. To maintain practically the same growth rate and feed economy of gains, the pigs in the cold consumed 4.0% of their body weight in food, while the pigs in the warm environment consumed only 2.5% of their body weight in food. This physiological response to cold is mediated through the thyroid gland (Ring, '42; Dempsey and Astwood, '43), the pituitary gland and the adrenals (Tyslowitz and Astwood, '42). The thyroid hormone is of fundamental importance in protein anabolism (Samuels, '48), and protein synthesis is associated with a decreased excretion of riboflavin in the urine (Oldham, Lounds and Porter, '47). Evidence of the same relationship will be given below for pigs. This association of protein retention and riboflavin retention suggests a greater requirement of the vitamin the greater the retention of dietary protein, and a greater requirement of riboflavin in a cold environment if such an environment favors protein retention. The latter point needs direct investigation.

In the third place, a cold environment affects the metabolism of fat, leading to the deposition, especially in the peripheral tissues, of fats with lower melting points and greater unsaturation (Söderlind, '40; Schmidt-Nielsen, '46). That riboflavin is involved in fat metabolism has been suspected for some time (Mannering, Lipton and Elvehjem, '41), but later (Potter, Axelrod and Elvehjem, '42) the evidence indicating a positive correlation between riboflavin and fat level in the diet was said to depend upon the nature of the carbohydrate in the diet. In an attempt to throw some light upon the nutrients whose metabolism involves riboflavin, we supplemented ordinary swine rations with fat (soybean oil),

TABLE 2

The effect of supplements of pure nutrients added to natural rations on the output of riboflavin in the urine of pigs

DAILY RATIONS	NUMBER OF PIGS	AVERAGE BODY WEIGHT	FEEDING PERIOD	TEMPERATURE OF ROOM	RIBOFLAVIN INTAKE PER DAY	RIBOFLAVIN IN URINE PER DAY	PROBABILITY OF A CHANCE OUTCOME
		kg	*wks.*	°*F.*	*mg*	*mg*	
Basal ration: 1.2–1.5 kg	4	93.5	2	85	4.71	2.18	
Same plus 0.37 kg oil [1]	4	96.2	2	85	5.18	1.13	0.0075
Basal ration: 0.8–1.0 kg	4	134.8	2	85	8.09	2.22	
Same plus 0.2 kg casein	4	135.7	2	85	8.08	1.42	0.032
Basal ration: 0.8–1.0 kg	3	105.8	4	85	5.56	2.32	
Same plus 0.8–1.0 kg glucose	3	109.9	4	85	5.06	1.46	0.27
Basal ration: 0.8–1.0 kg	4	107.6	4	40	4.88	1.09	
Same plus 0.8–1.0 kg glucose	4	109.9	4	40	4.91	0.95	0.24

[1] Soybean oil.

protein [3] and carbohydrate (glucose). These rations, and the unsupplemented ration, were fed to different groups of pigs, and the effects of the supplements on the riboflavin output in the urine were measured, with the results shown in table 2. Protein and fat supplements induced a significant depression in the riboflavin spillage in the urine, while glucose did not. The obvious conclusion is that protein and fat metabolism increases riboflavin requirements, though admittedly the evidence depends upon a particular, albeit reasonable, interpretation of the significance of urinary riboflavin.

If riboflavin is involved in fat metabolism, then the effect of cold in modifying the biochemical reactions leading to fat deposition may conceivably increase the requirement for riboflavin if this vitamin is involved in the enzyme systems inducing desaturation of fatty acids.

SUMMARY AND CONCLUSIONS

In two experiments involving 8 young pigs each, the riboflavin requirement was determined at environmental temperatures of 85°F. and 42°F., respectively. In each experiment the pigs were depleted of the vitamin by subsistence for three weeks on a semi-synthetic ration containing 0.2 to 0.4 p.p.m. of riboflavin. During subsequent experimental periods of 7 or 8 weeks at the respective temperatures, the pigs were fed 8 different levels of the vitamin, ranging from the basal level to 4 or 5 p.p.m.

The food consumption was equalized among all pigs in each experiment except when the pigs on the lower levels developed symptoms of deficiency. The adequacy of the riboflavin concentration was determined by the appearance and behavior of the pigs, the rate of growth and the blood picture.

The most significant morphological change in the blood associated with incipient riboflavin deficiency in the young pig is a marked increase in the concentration of neutrophilic granulocytes and in the percentage of these cells in the total leukocyte count.

[3] Labco casein.

The results clearly show that the riboflavin requirement is higher at the lower temperature, the requirements being approximately 1.2 (or somewhat less) p.p.m. at 85°F. and approximately 2.3 p.p.m. at 42°F. These values are equivalent to 0.55 and 1.59 mg of riboflavin per pound of feed and 1.4 and 4.2 mg per 100 lb. body weight, respectively.

Confirmation of this effect of temperature on the riboflavin requirement is found in the results of preliminary experiments on older pigs, revealing a consistently smaller spill-over of riboflavin in the urine, on the same diet, at 42°F. than at 85°F.

Auxiliary data on pigs show that supplements of protein and of fat added to a diet of natural feeds depress the output of riboflavin in the urine, indicating, though not proving, an increased requirement of the vitamin under conditions of increased assimilation of protein and fat. Similar tests with glucose supplements yielded negative results.

The riboflavin requirement of animals per unit of air-dry food seems to be independent of body size. Hence, this method of expressing the requirement is preferable to expression per unit of body weight.

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LITERATURE CITED

BIRD, F. H., V. S. ASMUNDSON, F. H. KRATZER AND S. LEPKOVSKY 1946 The comparative requirements of chicks and turkey poults for riboflavin. Poultry Sci., *25:* 47.

BOLTON, W. 1947 The riboflavin requirement of the White Wyandotte chick. II. Pure crystalline riboflavin as the vitamin supplement. J. Agr. Sci., *37:* 316.

CZACZKES, J. W., AND K. GUGGENHEIM 1946 The influence of diet on the riboflavin metabolism of the rat. J. Biol. Chem., *162:* 267.

DEMPSEY, E. W., AND E. B. ASTWOOD 1943 Determination of the rate of thyroid hormone secretion at various environmental temperatures. Endocrinology, *32:* 509.

ERSHOFF, B. H., AND A. D. ADAMS, JR. 1946 Effects of reduced caloric intake on leucocyte count of the rat. Proc. Soc. Exp. Biol. Med., *62:* 154.

FEDER, V. H., G. T. LEWIS AND H. S. ALDEN 1944 Studies on the urinary excretion of riboflavin. J. Nutrition, *27:* 347.

FENTON, P. F., AND G. R. COWGILL 1947 The nutrition of the mouse. I. A difference in the riboflavin requirements of two highly inbred strains. Ibid., *34:* 273.

GUERRANT, N. B., R. A. DUTCHER AND R. CROWTHERS 1937 Environmental temperature as a factor in the production and in the cure of rickets in the rat. Ibid., *14:* 471.

HEGSTED, D. M. 1948 The determination of minimum vitamin requirements for growth. Ibid., *35:* 399.

HORWITT, M. K., O. W. HILLS, C. C. HARVEY, E. LIEBERT AND D. L. STEINBERG 1949 Effects of dietary depletion of riboflavin. Ibid., *39:* 357.

HUGHES, E. H. 1940 The minimum requirement of riboflavin for the growing pig. Ibid., *20:* 233.

HUGHES, E. H., E. W. CRAMPTON, N. R. ELLIS AND W. J. LOEFFEL 1944 Recommended nutrient allowances for swine. Report of Committee on Animal Nutrition, National Research Council.

JOHNSON, B. C. 1948 Methods of Vitamin Determination. Burgess Publ. Co., Minneapolis.

KRIDER, J. L., S. W. TERRILL AND R. F. VAN POUCKE 1949 Response of weanling pigs to various levels of riboflavin. J. Animal Sci., *8:* 121.

MANNERING, G. J., M. A. LIPTON AND C. A. ELVEHJEM 1941 Relation of dietary fat to riboflavin requirement of growing rats. Proc. Soc. Exp. Biol. Med., *46:* 100.

MCCALL, K. B., H. A. WAISMAN, C. A. ELVEHJEM AND E. S. JONES 1946 A study of pyridoxine and pantothenic acid deficiencies in the monkey (Macaca mulatta). J. Nutrition, *31:* 685.

MILLS, C. A. 1943 Environmental temperatures and B-vitamin requirements: riboflavin and pyridoxine. Arch. Biochem., *2:* 159.

MORGAN, A. F., M. GROODY AND H. E. AXELROD 1946 Pyridoxine deficiency in dogs as affected by level of dietary protein. Am. J. Physiol., *146:* 723.

OLDHAM, H. G., F. JOHNSTON, S. KLEIGER AND H. HEDDERICH-ARISMENDI 1944 A study of the riboflavin and thiamine requirements of children of preschool age. J. Nutrition, *27:* 435.

OLDHAM, H. G., E. LOUNDS AND T. PORTER 1947 Riboflavin excretions and test dose returns of young women during periods of positive and negative nitrogen balance. Ibid., *34:* 69.

PATEK, A. J., JR., J. POST AND J. VICTOR 1941 Riboflavin deficiency in the pig. Am. J. Physiol., *133:* 47.

POTTER, R. L., A. E. AXELROD AND C. A. ELVEHJEM 1942 The riboflavin requirement of the dog. J. Nutrition, *24:* 449.

RIESEN, W. H., B. S. SCHWEIGERT AND C. A. ELVEHJEM 1946 The effect of the level of casein, cystine and methionine intake on riboflavin retention and protein utilization by the rat. Arch. Biochem., *10:* 387.

RING, G. C. 1942 The importance of the thyroid in maintaining an adequate production of heat during exposure to cold. Am. J. Physiol., *137:* 582.

SAMUELS, L. T. 1948 Nutrition and Hormones. C. C. Thomas, Publisher, Springfield, Illinois.

SCHAEFER, A. E., C. K. WHITEHAIR AND C. A. ELVEHJEM 1947 The importance of riboflavin, pantothenic acid, niacin and pyridoxine in the nutrition of foxes. J. Nutrition, *34:* 131.

SCHMIDT-NIELSEN, K. 1946 Melting points of human fats as related to their location in the body. Acta physiol. Scand., *12:* 123; through Chem. Abstr., *41:* 1304.

SHUKERS, C. F., AND P. L. DAY 1943 The effects of inanition and riboflavin deficiency upon the blood picture of the rat. J. Nutrition, *25:* 511.

SNELL, E. E., AND F. M. STRONG 1939 A microbiological assay for riboflavin. Ind. Eng. Chem., Anal. Ed., *6:* 346.

SNYDERMAN, S. E., K. C. KETRON, H. B. BURCH, O. H. LOWRY, O. A. BESSEY, L. P. GUY AND L. E. HOLT, JR. 1949 The minimum riboflavin requirement of the infant. J. Nutrition, *39:* 219.

SÖDERLIND, P. 1940 Factors affecting the consistency of hog fat. Kgl. Lantbruks-akad. Tid., *79:* 18; through Chem. Abstr., *34:* 4432.

STEGGERDA, F. R., AND H. H. MITCHELL 1946 Variability in the calcium metabolism and calcium requirements of adult human subjects. J. Nutrition, *31:* 407.

STRONG, F. M., AND L. E. CARPENTER 1942 Preparation of samples for microbiological determination of riboflavin. Ind. Eng. Chem., Anal. Ed., *14:* 909.

TOURTELLOTTE, D., AND W. E. BACON 1935 Variability of vitamin D response with temperature of environment. J. Nutrition, *10:* 683.

TYSLOWITZ, R., AND E. B. ASTWOOD 1942 The influence of the pituitary and adrenal cortex on resistance to low environmental temperatures. Am. J. Physiol., *136:* 22.

WEIR, D. R., R. W. HEINLE AND A. D. WELCH 1949 Role of pyridoxine in the production of leucocytes in normal and leukemic mice. Proc. Soc. Exp. Biol. Med., *72:* 457.

WILLIAMS, R. D., H. L. MASON, P. L. CUSICK AND R. M. WILDER 1943 Observations on induced riboflavin deficiency and the riboflavin requirement of man. J. Nutrition, *35:* 361.

WINTROBE, M. M. 1947 Clinical Hematology. Lea and Febiger, Philadelphia, 2nd ed.

WINTROBE, M. M., W. BUSCHKE, R. H. FOLLIS, JR. AND S. HUMPHREYS 1944 Riboflavin deficiency in swine with special reference to the occurrence of cataracts. Bull. Johns Hopkins Hosp., *75:* 102.

WINTROBE, M. M., R. H. FOLLIS, JR., M. H. MILLER, H. J. STEIN, R. ALCAYAGA, S. HUMPHREYS, A. SUKATA AND G. E. CARTWRIGHT 1943 Pyridoxine deficiency in swine. With particular reference to anemia, epileptiform convulsions and fatty liver. Ibid., *72:* 1.

THE NUTRITIVE VALUE OF LEGUME SEEDS

X. EFFECT OF AUTOCLAVING AND THE TRYPSIN INHIBITOR TEST FOR 17 SPECIES [1]

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Several legume seeds have been compared as to nutritive value when fed raw and after autoclaving. Osborne and Mendel ('17a) were the first to observe that a legume seed, the soybean, was improved by autoclaving. Since then, the legume seeds reported to be improved by autoclaving include the jack bean (Orru and Demel, '41), velvet bean (Sure and Read, '21; Finks and Johns, '21b), Adsuki bean (Johns and Finks, '21), lima bean (Finks and Johns, '21a; Everson and Heckert, '44; Richardson, '48), and common bean (Osborne and Mendel, '17b; Johns and Finks, '20; Everson and Heckert, '44; Richardson, '48). However, not all legume seeds have been found to be improved by autoclaving. Those reported not to be so improved include the peanut (Mitchell and Beadles, '35; Altschul et al., '48; Buss and Goddard, '48; Zimmermann, '48), chick pea (Blaizot, '47), sweet pea (Geiger et al., '33; Lewis et al., '48), mung bean (Milby, '45) and the common pea (Finks, Jones and Johns, '22; Woods et al., '43; Everson and Heckert, '44; Blaizot, '47; Jacquot et al., '47; Richardson, '48). Controversial reports are extant concerning the lentil (improved according to Jones and Murphy, '24, and not improved according to Blaizot, '47, and Jacquot et al., '47) and the black-eyed pea, *Vigna sinensis* (improved according to

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Finks, Jones and Johns, '22, and Sherman, '41, and not improved according to Richardson, '48).

Ham, Sandstedt and Mussehl ('45) advanced the hypothesis that the trypsin inhibitor found in raw soybeans, and destroyed by autoclaving, was the cause of the lower nutritive value of raw soybeans compared with autoclaved ones. A previous publication by the present authors (Borchers and Ackerson, '47) reported a survey of 30 species of legume seeds for the trypsin inhibitor; 17 were found to contain the inhibitor, 13 not to contain it. Since then, Klose, Greaves and Fevold ('48) have reported that a lima bean preparation inhibited the growth of rats fed acid-hydrolyzed casein, and Westfall, Bosshardt and Barnes ('48) have reported that a soybean fraction inhibited the growth of rats fed enzyme-hydrolyzed casein. Results from this laboratory (Borchers, Ackerson and Mussehl, '48) have shown that a purified soybean trypsin inhibitor preparation does not inhibit the growth of chicks and rats.

The objects of the following investigation were: first, to observe the effect of autoclaving on the nutritive value of additional legume seeds for the rat; and second, to note whether improvement after autoclaving was correlated with the occurrence of the trypsin inhibitor, as is the case in the soybean.

MATERIALS

The legume seeds used in this study were obtained from a number of sources.[2]

The seeds were first ground to pass a 1-mm screen. The portion to be autoclaved was placed in shallow pans at a thickness

[2] Raw peanuts and lentils were purchased locally. Partridge peas, lespedeza and vetch *(Vicia sativa)* were contributed by the Department of Agronomy, Nebraska Agricultural Experiment Station, guar by Mr. D. C. Aepli, Department of Agronomy, Arizona Agricultural Experiment Station, and mung beans by Dr. L. L. Ligon, Department of Agronomy, Oklahoma Agricultural Experiment Station. Jack beans were purchased from the Arlington Chemical Co., Yonkers, N. Y., velvet beans from T. W. Wood and Sons, Richmond, Va., horse beans from the W. A. Burpee Co., Clinton, Iowa, and black-eyed peas *(Vigna sinensis)* from the H. G. Hastings Co., Atlanta, Ga., and the Aggler-Musser Seed Co., Los Angeles, Calif.

not exceeding 0.5 cm. Autoclaving was carried out at 15 pounds' pressure for 30 minutes, except where noted in table 1. After autoclaving, the meal was spread before a fan and allowed to dry at room temperature. The peanuts were extracted with petroleum ether following grinding.

EXPERIMENTAL

The rats used in this study were of the Sprague-Dawley strain. They were placed on experimental rations at weaning, when they were 20 to 23 days of age and at a weight of approximately 35 gm. They were housed in individual, screen-bottom cages with food and water available ad libitum. Feeding was continued for 20 days. The total food consumption of each animal was recorded. The results are expressed as average grams of gain per day and as grams of gain per gram food consumed. The rats were distributed so that 4 males and 4 females were placed on the raw legume ration and a litter mate of the same sex and initial weight was placed on the autoclaved legume ration. The composition of the rations was as follows: raw or autoclaved legume seed sufficient to supply 12 gm protein ($N \times 6.25$, protein contents given in table 1), 0.6 gm DL-methionine, 2 gm salt mixture (Hubbell, Mendel and Wakeman, '37), 0.5 gm choline chloride, 0.5 mg thiamine HCl, 1.25 mg Ca pantothenate, 2.5 mg niacin, 0.125 mg pyridoxine HCl, 0.5 mg riboflavin, 5 gm cod liver oil, 10 gm lard, and corn starch sufficient to make 100 gm.

RESULTS

Of 11 legumes fed in this study, the nutritive value of 5 was improved by autoclaving as judged by rat growth when the legume served as the only source of protein at a 12% level. Six legumes were not improved. The results are presented in table 1. Of the 17 legume seeds included in this and other studies, 8 were improved by autoclaving; 5 of these contain the trypsin inhibitor, three do not. Nine of these 17 legume seeds were not improved by autoclaving; 6 of these contain the trypsin inhibitor, three do not. These are listed in table 2.

TABLE 1

Effect of autoclaving on the nutritive value of 11 legume seeds for the rat

LEGUME [1]	PROTEIN (N × 6.25)	RAT GROWTH GM/DAY ± SE [2] GM GAIN/GM FOOD CONSUMED		
		Raw	Autoclaved [3]	"t" value [4]
	%			
Arachis hypogaea Peanut	52.5 [5]	1.30 ± 0.11 0.22	1.40 ± 0.13 0.23	0.588 [6]
Canavalia ensiformis Jack bean	26.0	0.30 ± 0.11 0.10	1.40 ± 0.13 0.31	6.410 [7]
Chamaecrista fasiculata Partridge pea	36.2	2.72 ± 0.16 0.33	2.61 ± 0.22 0.32	0.409 [6]
Cyamopis psoraloides Guar bean	23.6	0.29 ± 0.13 0.06	0.27 ± 0.06 0.06	0.143 [6]
Lens esculenta Lentil	27.4	0.36 ± 0.05 0.10	0.58 ± 0.07 0.15	2.464 [8]
Lespedeza stipulacea Lespedeza	35.5	2.98 ± 21 0.36	3.26 ± 0.22 0.39	0.933 [6]
Mucana deeringianum Velvet bean	24.5	— 0.26 ± 0.05 — 0.08	1.25 ± 0.11 0.30	12.607 [7]
Phaseolus aureus Mung bean	29.8	1.89 ± 0.14 0.33	2.08 ± 0.19 0.34	0.792 [6]
Vicia faba Horse bean	25.0	1.58 ± 0.11 0.22	2.12 ± 0.16 0.31	2.842 [8]
Vicia sativa Common vetch	27.7	0.41 ± 0.07 0.10	0.36 ± 0.12 0.09	0.357 [6]
Vigna sinensis Black-eyed pea	22.3			
Sample 1		1.41 ± 0.14 0.27	2.20 ± 0.19 0.35	3.379 [7]
Sample 2		0.87 ± 0.08 0.20	1.29 ± 0.10 0.27	3.333 [7]
Autoclaved 15 min.		1.01 ± 0.11 0.21	1.56 ± 0.07 0.29	4.231 [7]
Autoclaved 60 min.		above	1.51 ± 0.10 0.28	3.333 [7]
Methionine omitted		0.24 ± 0.04 0.06	0.87 ± 0.11 0.16	5.431 [7]

[1] Nomenclature according to Graham ('41).

[2] Eight rats, 4 females and 4 males on each ration, paired as to litter and initial weight between rations, fed for 20 days.

[3] Autoclaved at 15 pounds' pressure for 30 minutes, except where noted for black-eyed pea.

[4] The "t" value according to Snedecor ('46).

[5] Protein in the peanut after extracting with petroleum ether.

[6] Not significantly different.

[7] Significant at the 1% level.

[8] Significant at the 5% level.

DISCUSSION

The results of this investigation show that the legume seeds studied are approximately equally divided as to those which are and are not improved in nutritive value by autoclaving. Further, there clearly exists no correlation between improvement in nutritive value after autoclaving and the presence or absence of the trypsin inhibitor in the raw legume seed. Hence, the trypsin inhibitor test does not indicate whether or not the legume seed will be improved by autoclaving.

The feeding results in this study agree with those available in the literature, with two exceptions. The reports of Blaizot

TABLE 2

Effect of autoclaving on the nutritive value of legume seeds and trypsin inhibitor test

IMPROVED BY AUTOCLAVING		NOT IMPROVED BY AUTOCLAVING	
Inhibitor [1] positive	Inhibitor [1] negative	Inhibitor [1] positive	Inhibitor [1] negative
Velvet bean	Jack bean	Peanut	Guar
Lima bean	Lentil	Partridge pea	Common pea
Common bean	Horse bean	Chick pea	Common vetch
Soybean		Sweet pea	
Black-eyed pea		Lespedeza	
		Mung bean	

[1] Trypsin inhibitor test from Borchers and Ackerson ('47).

('47) and Jacquot et al. ('47) that the common lentil is not improved by heating are contradicted, and the earlier report of Jones and Murphy ('24) is confirmed. The recent report of Richardson ('48) that the black-eyed pea *(Vigna sinensis)* is not improved by heating was also contradicted. More extensive studies on the black-eyed pea, as is shown in table 1, have failed to reveal any effect on the final outcome by autoclaving for periods of 15, 30, or 60 minutes; omission of methionine from the ration; or use of seeds from two different sources. Richardson autoclaved at 15 pounds' pressure for 30 minutes and omitted methionine from the ration.

SUMMARY

The seeds of 11 species of legumes were fed, raw and autoclaved, to rats as the sole source of protein at a 12% protein level. The jack bean, lentil, velvet bean, horse bean, and black-eyed pea were improved by autoclaving, while the peanut, partridge pea, guar, lespedeza, mung bean, and common vetch were not improved. Of a total of 17 species of legume seeds investigated in this and other studies, as listed in table 2, 8 were reported to be improved by autoclaving while 9 were not. No correlation was observed between the effect of autoclaving on nutritive value and the presence or absence of the trypsin inhibitor in the raw legume seed.

LITERATURE CITED

ALTSCHUL, A. M., G. W. IRVING, W. F. GUILBEAU AND H. C. SCHAEFER 1948 The nutritive value of peanut cake, meal, protein and nonprotein residue for chicks. Poultry Sci., *27:* 402.

BLAIZOT, J. 1947 Effects of heat-treatment upon the food value of some legumes. Bull. Soc. sci. hyg. aliment., *35:* 23; from Chem. Abst., *42:* 696f, 1948.

BORCHERS, R., AND C. W. ACKERSON 1947 Trypsin inhibitor. IV. Occurrence in seeds of the *Leguminosae* and other seeds. Arch. Biochem., *13:* 291.

BORCHERS, R., C. W. ACKERSON AND F. E. MUSSEHL 1948 Trypsin inhibitor. VIII. Growth inhibiting properties of a soybean trypsin inhibitor. Ibid., *19:* 317.

BUSS, L. W., AND V. R. GODDARD 1948 Effect of heat upon the nutritive values of peanuts. I. Protein quality. Food Res., *13:* 506.

EVERSON, G., AND A. HECKERT 1944 The biological value of some leguminous sources of protein. J. Am. Diet. Assn., *20:* 81.

FINKS, A. J., AND C. O. JOHNS 1921a Studies in nutrition. VI. The nutritive value of the proteins of lima bean, *Phaseolus lunatus.* Am. J. Physiol., *56:* 205.

———— 1921b Studies in nutrition. IX. The nutritive value of the proteins from the Chinese and Georgia velvet beans. Ibid., *57:* 61.

FINKS, A. J., D. B. JONES AND C. O. JOHNS 1922 The role of cystine in the dietary properties of the proteins of the cow-pea, *Vigna sinensis,* and of the field pea, *Pisum sativum.* J. Biol. Chem., *52:* 403.

GEIGER, B. J., H. STEENBOCK AND H. T. PARSONS 1933 Lathyrism in the rat. J. Nutrition, *6:* 427.

GRAHAM, E. N. 1941 Legumes for Erosion Control and Wildlife. U. S. Dept. Agr., Misc. Publ. 412.

HAM, W. E., R. M. SANDSTEDT AND F. E. MUSSEHL 1945 The proteolytic inhibiting substance in the extract from unheated soy bean meal and its effect upon growth in chicks. J. Biol. Chem., *161:* 635.

HUBBELL, R. B., L. B. MENDEL AND A. J. WAKEMAN 1937 A new salt mixture for use in experimental diets. J. Nutrition, *14:* 273.

JACQUOT, R., J. MATET AND O. FRIDENSON 1947 Effect of heat-treatment on foods as sources of proteins. Ann. nutrition et aliment., *1:* 157; from Chem. Abst., *41:* 6633i.

JOHNS, C. O., AND A. J. FINKS 1920 Studies in nutrition. II. The role of cystine in nutrition as exemplified by nutrition experiments with the proteins of the navy bean, *Phaseolus vulgaris.* J. Biol. Chem., *41:* 379.

———— 1921 Studies in nutrition. VII. The nutritive value of the Adsuki bean, *P. angularis.* Am. J. Physiol., *56:* 208.

JONES, D. B., AND J. C. MURPHY 1924 Cystine deficiency and vitamin content of the lentil, *Lens esculenta* Moench. J. Biol. Chem., *59:* 243.

KLOSE, A. A., J. D. GREAVES AND H. L. FEVOLD 1948 Inadequacy of proteolytic enzyme inhibition as explanation for growth depression by lima bean protein fractions. Science, *108:* 88.

LEWIS, H. B., R. S. FAJANS, M. B. ESTERER, C.-W. SHEN AND M. OLIPHANT 1948 The nutritive value of some legumes. Lathyrism in the rat. The sweet pea *(Lathyrus odoratus), Lathyrus sativus, Lathyrus cicera* and some other species of *Lathyrus.* J. Nutrition, *36:* 537.

MILBY, T. T. 1945 Mung beans as a source of protein for turkey poults. Proc. Okla. Acad. Sci., *25:* 50.

MITCHELL, H. H., AND J. R. BEADLES 1935 Nuts fail as adequate substitutes for meat. Illinois Agr. Expt. Sta., 47th Ann. Rep., 1933–1934: p. 80.

ORRU, A., AND V. C. DEMEL 1941 Physiological and anatomical-pathological observations on rats fed the seeds of *Canavalia ensiformis.* Quaderni nutriz., *7:* 273; from Chem. Abst., *36:* 3532, 1942.

OSBORNE, T. B., AND L. B. MENDEL 1917a The use of soy bean as food. J. Biol. Chem., *32:* 369.

———— 1917b Continuation and extension of work on vegetable proteins. Carnegie Inst. of Washington Yearbook, *16:* 324.

RICHARDSON, L. R. 1948 Southern pea and other legume seeds as a source of protein for the growth of rats. J. Nutrition, *36:* 451.

SHERMAN, W. C. 1941 Comparison of the nutritive value of the protein of cowpeas and soybeans. Alabama Agr. Expt. Sta., 51st Ann. Rep., p. 25.

SNEDECOR, G. W. 1946 Statistical Methods. Iowa State College Press, Ames.

SURE, B., AND J. W. READ 1921 Biological analysis of the seed of the Georgia velvet bean *Stizolobium deeringianum.* J. Agr. Res., *22:* 5.

WESTFALL, R. J., D. K. BOSSHARDT AND R. H. BARNES 1948 Influence of crude trypsin inhibitor on utilization of hydrolyzed protein. Proc. Soc. Exp. Biol. Med., *68:* 498.

WOODS, E., W. M. BEESON AND D. W. BOLIN 1943 Field peas as a source of protein for growth. J. Nutrition, *26:* 327.

ZIMMERMANN, G. 1948 Biological examination of the influence of drying on the nutritive value of the nitrogen-containing substance in feed and foodstuffs. Mitt. Gebiete Lebensm. Hyg., *39:* 71; from Chem. Abst., *42:* 6956h.